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# THE AMERICAN REVIEW OF TUBERCULOSIS

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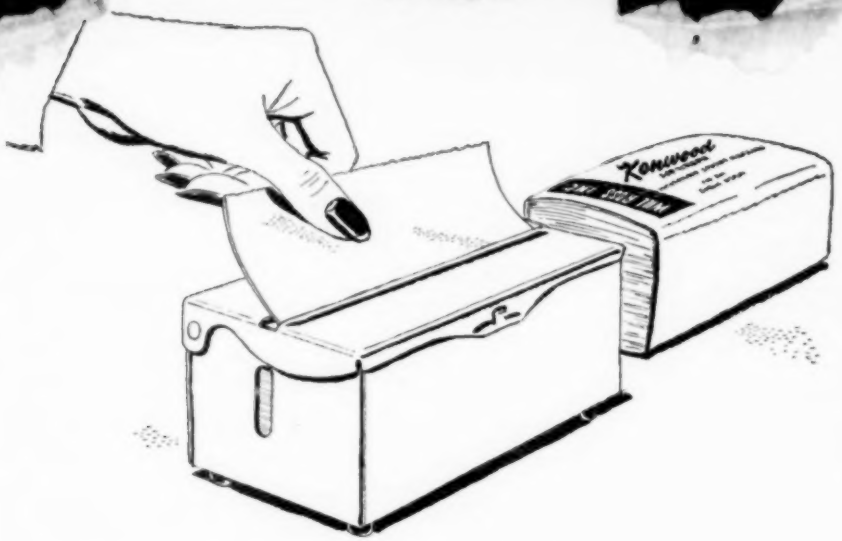


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VOLUME 66 *September, 1952* NUMBER 3

# LUNG IMMOBILIZER THERAPY IN PULMONARY TUBERCULOSIS[1]

## A Clinical Study

ROGER A. HEMPHILL, GEORGE C. OWEN, AND JOHN D. STEELE

(Received for publication April 7, 1952)

### INTRODUCTION

In 1940 Barach (1, 2) applied the principle of Thunberg's barospirator (3) to the treatment of tuberculosis. In brief, the apparatus consists of a chamber, somewhat like a Drinker Respirator, with alternating pressure but with the whole body enclosed. Air enters the head end, and a movable partition "produces differential pressure in the head compartment" which "in the compression (positive) cycle of air is adequate to overcome the resistance of the tracheobronchial tree, thus allowing equal air pressure to be simultaneously applied to the inner and outer surfaces of the chest, as well as to the upper and lower surfaces of the diaphragm" (4). In this way intra- and extra-thoracic pressures remain in dynamic equilibrium. Mechanical breathing takes the place of natural breathing. In contrast to natural breathing (and most respirators), however, in which air is moved by volume with resulting motion of the chest and moderate pressure change, in the immobilizer air is moved by change in pressures without change in volume. Emptying and filling of a rubber balloon as opposed to an oxygen tank provide a fair analogy except that, in the lung immobilizer, volume is kept constant, not by a rigid chest wall, but by applying equal pressure without while pressure is raised within. For details, the original papers and a report of the Committee on Therapy of the American Trudeau Society should be consulted (2, 5, 6, 7, 8).

It has been reported that tuberculous patients treated in the chamber have improved, that sputum has "converted," and that cavities have closed (4, 5, 9). Results have been interpreted as so beneficial that "in fact, the trial of properly managed immobilizing chamber therapy in preference to . . . thoracoplasty might (induce) clinical recovery without impairment of lung function or structure and without the risk attendant on other methods . . ." (10) and "the procedure . . . will now appear to be indicated in many patients with this disease (tuberculosis) in preference to either pneumothorax or thoracoplasty" (11).

[1] From the Veterans Administration Center, Wood, Wisconsin; and Marquette University School of Medicine, Milwaukee, Wisconsin.

The present report is concerned with the results obtained by use of the immobilizer in a series of 18 tuberculous patients studied in this clinic.

## PLAN OF INVESTIGATION

Inasmuch as marked improvement (5, 6) had been reported in patients treated three and one-half to four months for six to eight hours a day, it was decided, since the present investigators had only one chamber available, that the patients would be treated for eight hours a day. In this way, it was possible to treat 3 patients in the same twenty-four-hour span and on a four-month interval and to treat 9 patients for the interval stated within one year. Although six days a week have been considered adequate treatment (6), it was decided to adhere to seven, and with rare exceptions this proved possible.

The following criteria were set up for the selection of patients and were considered to offer an objective standard for measuring the merits of this type of treatment. In general, it was possible to follow these criteria except for the individual modifications mentioned below.

### *Selection of Cases*

Lesions were chosen which were frankly active as proved by roentgenographic and bacteriologic studies.

An attempt was made to provide a minimum four-month period of observation before treatment; during this time the lesions were stationary or progressive. If possible, the patients were maintained on strict bed rest during the observation period.

The lesions were moderately or far advanced in extent.

The presumed life expectancy of each patient was one year or longer in the absence of any special treatment.

No collapse treatment was started during the four-month observation period. Any collapse therapy in progress was continued unchanged during the treatment and follow-up period.

No antituberculous chemotherapy had been administered for six months prior to entry into the chamber. This provision was neglected if the lesion had been getting worse since the discontinuation of chemotherapy.

Cavitary disease received preference.

Bronchoscopy, planigrams, and pulmonary function studies were performed at the start of treatment whenever possible.

### *Observations During Treatment in the Lung Immobilizing Chamber*

Monthly roentgenograms, sputum and gastric studies, and sedimentation rates were made, and other laboratory work as indicated. No other therapy was started during the treatment period; this refers particularly to chemotherapy and collapse therapy.

Treatment was given for eight hours daily over a period of four months. This may be divided into two four-hour periods daily.

The patients were maintained on bed rest during the remainder of the day or on the same amount of activity as during the observation period.

Bronchoscopy, planigrams, and pulmonary function studies were repeated at the completion of treatment when possible.

### *Follow-up Observations*

Monthly roentgenograms were made for four months and other examinations as indicated.

No new therapy was started during a four-month follow-up period. This refers particularly to chemotherapy and collapse therapy.

The patients were continued on the same degree of bed rest as was present during the observation and treatment periods.

The general plan was followed as closely as practicable. In order to save time, however, patients were selected for treatment in the chamber before patients already under treatment had completed their courses. In at least one instance, as will be shown, a cavity which had been stable for several months suddenly began spontaneous contraction between the time of pretreatment selection and the time treatment actually was begun. Follow-up examinations also, while obtained in every instance, could not be obtained always at the intervals indicated because of transfer to other hospitals, irregular discharge, et cetera. Nevertheless, this did not significantly affect the evaluation of results.

In the period of treatment, 18 patients were started on this type of therapy. Within two weeks, 2 of the patients declined further therapy because of distressing aggravation of paranasal sinusitis and were dropped from the study. Sixteen patients, therefore, are being reported, although one was removed from the chamber because of a serious spread of disease at the end of three months of therapy.

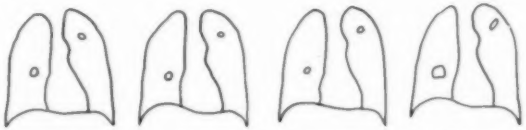
The roentgenographic course of the individual cases is presented in the following manner: Tracing paper was superimposed over the actual roentgenogram, the chest was outlined, and the cavities concerned were very carefully traced to actual size. These tracings were then photographed together with symmetrical reduction, so that comparison can easily be made.

## RESULTS

The lesions of 3 patients showed significant improvement. The patient of *Case 1* had had a stable roentgenogram for some months prior to selection for treatment, but, approximately one month later, on beginning treatment, it was found that significant contraction in cavity size had already occurred. This contraction continued throughout the course of treatment and, on return to his home hospital, a localized resection was done. This is the only patient of the 16 whose pulmonary secretions became negative for *M. tuberculosis* on culture. The patient of *Case 2* showed significant improvement under treatment and this improvement has been maintained, but the patient's secretions are still positive for *M. tuberculosis* on culture. In *Case 3*, contraction of cavity occurred during treatment with prompt increase in size after treatment was ended. In the remaining 13 patients, the lesions of 9 were considered to show no significant change, and in 4 the lesions actually became worse under treatment (*see* illustrations). It may be noted that bed rest during treatment was equivalent to, often greater than, that before or after treatment in the immobilizer.

One patient (*Case 8*) had a cavernostomy. Advantage was taken of this fact by Dr. Emil Rothstein to measure intracavitary pressure changes during chamber therapy. A catheter with rather rigid walls was introduced into the chamber and attached to a manometer outside. The lower end of the catheter was clamped off, and with the chamber in operation no fluctuation was noted on the manometer. The catheter was then unplugged and sealed snugly within the cavernostomy. The manometer then reflected faithfully the pressure changes occurring in the chamber itself, showing that in this instance there was no significant bronchial obstruction.

PHOTOGRAPH OF TRACINGS OF ROENTGENOGRAMS

| | *Selection Film* | *Start of Treatment* | *End of Treatment* | *Follow-up* |
|---|---|---|---|---|
| | | | | |
| Case 1. (E. H.) ..... | 7/ 6/50 | 8/ 4/50 | 12/12/50 | 7/31/51 |
| | | | | |
| Case 2. (J. H.) ..... | 9/15/49 | 11/30/49 | 3/28/50 | 5/25/50 |
| |  | | | |
| Case 3. (L. P.) ..... | 10/21/49 | 12/ 7/49 | 3/29/50 | 5/10/50 |
| | | | | |
| Case 4. (R. A.) ..... | 12/20/48 | 12/ 8/49 | 3/28/50 | 4/ 6/51 |

Case 1. (E. H.) After a period of stability, cavity on left began to improve between time of selection and time of beginning treatment.

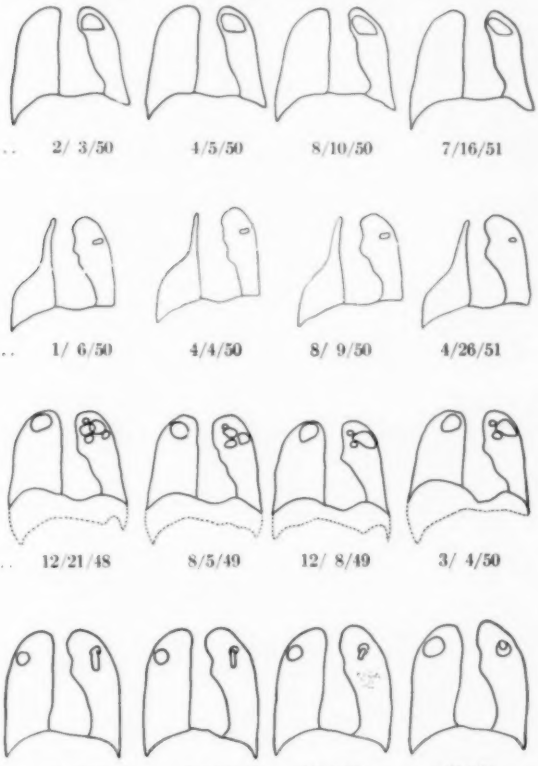
Case 2. (J. H.) Cavity improved while in chamber: secretions still positive for *M. tuberculosis*.

Case 3. (L. P.) Right mid-chest cavity the target of therapy; improvement on therapy; worsening after.

Case 4. (R. A.) Left lung opaque after pneumothorax. No results on right from chamber treatment.

PHOTOGRAPH OF TRACINGS OF ROENTGENOGRAMS

| | *Selection Film* | *Start of Treatment* | *End of Treatment* | *Follow-up* |
|---|---|---|---|---|
| Case 5. (G. B.) | 2/ 3/50 | 4/5/50 | 8/10/50 | 7/16/51 |
| Case 6. (R. B.) | 1/ 6/50 | 4/4/50 | 8/ 9/50 | 4/26/51 |
| Case 7. (K. C.) | 12/21/48 | 8/5/49 | 12/ 8/49 | 3/ 4/50 |
| Case 8. (E. Lng) | 5/ 9/49 | 8/5/49 | 12/ 2/49 | 6/20/51 |



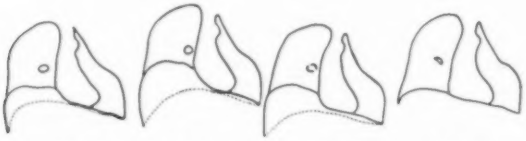
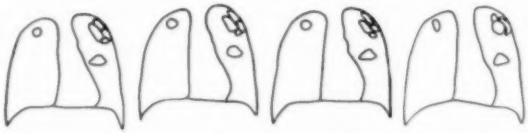
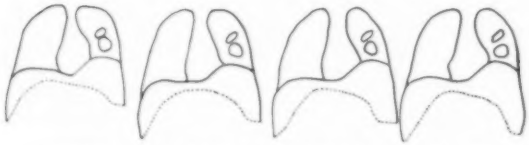
Case 5. (G. B.) Patient had declined surgery for left apical cavity.

Case 6. (R. B.) Surgery not feasible for left apical cavity.

Case 7. (K. C.) Dotted lines indicate pneumoperitoneum. Chamber control of either apex would have permitted surgery for the other.

Case 8. (E. Lng) Cavity, right; cavernostomy, left (*see* roentgenograms)

PHOTOGRAPH OF TRACINGS OF ROENTGENOGRAMS

| | *Selection Film* | *Start of Treatment* | *End of Treatment* | *Follow up* |
|---|---|---|---|---|
|  | | | | |
| Case 9. (G. S.) | 6/28/50 | 8/10/50 | 12/12/50 | 8/24/51 |
| | | | | |
| Case 10. (S. R.) | 5/26/50 | 8/ 4/50 | 12/12/50 | 4/13/51 |
|  | | | | |
| Case 11. (J. S.) | 3/ 1/50 | 4/ 5/50 | 8/10/50 | 4/13/51 |
|  | | | | |
| Case 12. (W. M.) | 5/10/51 | 5/18/51 | 9/21/51 | 1/ 9/52 |

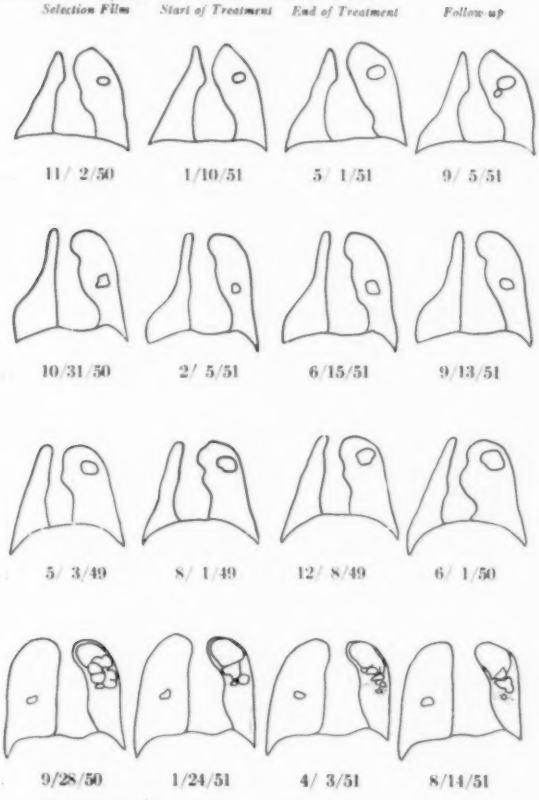
Case 9. (G. S.) Target area right lower lobe cavity.

Case 10. (S. R.) Patient refused surgery.

Case 11. (J. S.) Chamber control of right apical cavity would have permitted surgery on left.

Case 12. (W. M.) Dotted line indicates pneumoperitoneum.

PHOTOGRAPH OF TRACINGS OF ROENTGENOGRAMS




Case 13. (E. B.) Pneumonectomy on right, very recent cavity on left (*see* roentgenograms).

Case 14. (T. M.) Pneumoperitoneum failure for left lower lobe cavity.

Case 15. (E. Lgk) Pneumoperitoneum failure.

Case 16. (R. N.) Control of cavity on right would have permitted surgery on left; significant spread of disease required curtailment of treatment period.

Case 8. (E. Lng) May 9, 1949 Case 8. (E. Lng) August 5, 1949

Case 8. (E. Lng) December 2, 1949 Case 8. (E. Lng) June 20, 1951

Sample film reproductions: The preceding tracings were made from the roentgenograms themselves; only a few films lend themselves to clear reproduction.

Case 13. (E. B.)..... November 2, 1950

Case 13. (E. B.) January 10, 1951

Case 13. (E. B.)..... May 1, 1951

Case 13. (E. B.)..... September 5, 1951

Sample film reproductions: The preceding tracings were made from the roentgenograms themselves; only a few films lend themselves to clear reproduction.

SUMMARY

In 16 patients with chronic pulmonary tuberculosis treated in the lung immobilizing chamber for eight hours a day for four months, the lesions of 4 became worse, 9 showed no change, and 3 showed improvement. Improvement in one of the 3 had begun just prior to therapy; in another, it was not maintained after therapy.

SUMARIO

*La Terapéutica Inmovilizadora del Pulmón en la Tuberculosis Pulmonar: Estudio Clínico*

De 16 enfermos con tuberculosis pulmonar crónica tratados en la cámara inmovilizadora del pulmón durante ocho horas diarias por espacio de cuatro meses, las lesiones de 4 empeoraron, las de 9 no se alteraron y las de 3 revelaron mejoría. La mejoría en uno de los últimos había comenzado precisamente antes de la terapéutica; en otro no se mantuvo después de aquella.

RESUME

*Thérapie par immobilisation du poumon dans la tuberculose pulmonaire: Une étude clinique*

Chez 16 malades atteints de tuberculose pulmonaire chronique, traités dans un appareil pour l'immobilisation pulmonaire, pendant huit heures par jour pendant quatre mois, les lésions de 4 d'entre eux se sont aggravées, 9 n'ont montré aucune modification et 3 ont présenté une amélioration. Chez un de ces trois derniers malades le début de l'amélioration s'était manifesté aussitôt avant le traitement; chez un autre l'amélioration n'a pas persisté après le traitement.

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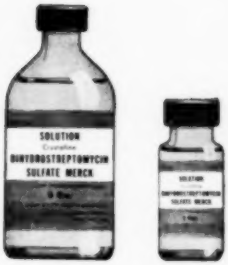


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# STREPTOMYCIN AND DIHYDROSTREPTOMYCIN IN THE TREATMENT OF TUBERCULOUS EMPYEMA[1]

## I. Experimental Studies of the Drug Concentrations Attained with Various Vehicles

I. STÅHLE

(Received for publication February 11, 1952)

### INTRODUCTION

During intramuscular streptomycin treatment, streptomycin is distributed into the pleural fluid. The concentration in the pleural fluid does not seem to reach particularly high values. It is reported in various publications that, with a dose of 0.6 to 2.0 gm. administered intramuscularly, a concentration of streptomycin of 1 to 18 $\gamma$ per ml. has been found in the pleural fluid at a time when the concentration in the blood was 15 to 20 $\gamma$ per ml. A measurable amount of streptomycin does not seem to be present in the pleural fluid until one-half to one hour after the injection (1, 7, 8, 13, 16, 17). According to Pulaski and Sprinz (11), the concentration in the pleural fluid is approximately one-fourth to one-half of the blood concentration prevailing at the same time during the first hours following the intramuscular injection. In investigations reported by Christensen (4), it was shown that, after a single intramuscular dose of 0.5 gm. of streptomycin, the concentration of drug in the pleural fluid remained fairly constant for two to twelve hours, approximately 5 $\gamma$ per ml.

In connection with the intrapleural injection of 1 gm. of streptomycin, Christensen (4) found in two cases that, three or four days after the instillation of the drug, the concentration in the pleural fluid remained as high as 150 to 200 $\gamma$ per ml.

Levin and his collaborators (5) stated that, with 1 gm. of dihydrostreptomycin given intramuscularly, the concentration of drug in the pleural fluid was as high as, or higher than, the blood concentration (two cases, the first one with a concentration in the pleural fluid of 2.75 $\gamma$ per ml. and in the blood of 3.35 $\gamma$ per ml. after eighteen hours; the second one, 5.8 $\gamma$ per ml. and 3.3 $\gamma$ per ml., respectively, after twenty-four hours). In their opinion, the findings indicated that dihydrostreptomycin passes fairly freely from blood to pleural fluid.

Empyema fluid usually has a slight acid or neutral reaction of approximately pH 6.8 to 7.0, and in this environment the effect of streptomycin is reduced. The best effect of streptomycin is attained at pH $\geqq$ 7.8 (4.16). Attempts have been made, therefore, especially by French and Italian investigators, to alkalinize the pus in cases of empyema in order to enhance the effect of streptomycin (3, 9, 12, 14). Rinsing with a 2 to 4 per cent sodium bicarbonate solution or with twentieth-normal sodium hydroxide solution buffered with twentieth-molar borax solution has been tried, but only a transient pH increase was obtained. Posternak (10) succeeded in maintaining a pH of approximately 7.5 by the intrapleural application of 2.5 to 4.0 per cent polyvinyl alcohol buffered with 0.1 per cent

[1] From the Kolmården Sanatorium, Sweden.

borax solution. Posternak assumed that polyvinyl alcohol, a gel with molecules of considerable size, delayed the absorption of the streptomycin.

The object of the present study was, first, to ascertain the amount of streptomycin and dihydrostreptomycin which is required to maintain a therapeutically effective concentration in the pleural fluid; and, second, to find a suitable vehicle which would keep the pH at an adequate level for maintaining the full effect of the drug.

## EXPERIMENTAL

### *Methods and Procedures*

*Test objects:* The experiments were carried out in 7 cases of tuberculous empyema, 2 of which were of the extrapleural type (for details *see* reference 15). Forty-four series of experiments were carried out.

*Methods:* The streptomycin and dihydrostreptomycin concentrations in the blood and in the pleural fluid were determined by a modification of the method described by Loo and collaborators (6). A number of filter paper tablets, containing 0.01 ml. of a solution of streptomycin or dihydrostreptomycin in various concentrations, were placed on a large agar plate (the agar medium prepared with beef extract, peptone, and saccharose). Between these tablets were placed other filter tablets containing 0.01 ml. of the samples. The test organism used was *Bacillus subtilis*. After incubation at 32°C. for seventeen to twenty-four hours, the inhibition zones of the unknown samples and the streptomycin or dihydrostreptomycin concentrations were calculated.

The pH of the pleural fluid was determined with Accutint paper. With this method, the accuracy is approximately 0.1 to 0.2 pH when two or more indicator papers with different pH ranges are used.

*Vehicles:*

Physiological saline (0.9 per cent sodium chloride)

Cellugel I

| | |
|---|---|
| Sodium carboxymethyl cellulose | 10.0 gm. |
| Sodium chloride | 8.0 gm. |
| Nipagin[3] | 1.0 gm. |
| Sterile water | 1,000.0 ml. |

1 Normal sodium hydroxide to make pH = 9.5

Cellugel II

| | |
|---|---|
| Sodium carboxymethyl cellulose | 10.0 gm. |
| Disodium phosphate | 16.7 gm. |
| Nipagin[3] | 1.0 gm. |
| Sterile water | 1,000.0 ml. |

pH = 8.15

*Preparations:*

Streptomycin: The preparation used was a complex salt of streptomycintrichloride and calcium chloride, containing 0.730 gm. base per gm.

Dihydrostreptomycin: The preparation used was bis-dihydrostreptomycintrisulfate, containing 0.780 gm. base per gm.

Nipagin is added in order to better conserve the solution.

*Procedure:* After all the pus had been emptied and the pleural cavity had been rinsed with 0.9 per cent sodium chloride (NaCl) until a clear fluid was obtained, 300 ml. of the vehicle (0.9 per cent NaCl, cellugel I, or cellugel II), to which

[3] Nipagin is methylparabenzoate (methylparaben in the American Pharmacopea).

streptomycin, 1.0 gm., or varying amounts of dihydrostreptomycin, 0.5 to 1.0 gm., were added, were instilled into the pleural cavity. Then streptomycin or dihydrostreptomycin was added to the pleural fluid daily or once every two days for a total period of ten days.

The concentration of streptomycin or dihydrostreptomycin in the pleural fluid was determined immediately after the first application of vehicle and drug and immediately prior to each instillation of the drug. The pH was determined every day or every second day during the same period, the first value being taken twenty-four hours after the administration into the pleural cavity of vehicle and drug.

In a series of experiments, dihydrostreptomycin was administered as one single dose (1.0 gm.) into the vehicle (0.9 per cent NaCl or cellugel II). The concentrations of dihydrostreptomycin were determined immediately after the application and then daily for three or four days.

Thus, streptomycin and dihydrostreptomycin were only administered locally into the pleural fluid in this study.

## RESULTS

### *Significance of the Vehicle*

*Streptomycin:* In the first series of experiments, 1 gm. of streptomycin was given initially with 300 ml. of 0.9 per cent NaCl; then 1 gm. of streptomycin was instilled daily for four days. The concentration of streptomycin in the pleural fluid dropped rapidly, after a high initial value of 2,500 $\gamma$ per ml., to 50 to 100 $\gamma$ per ml. after forty-eight to seventy-two hours. The results are illustrated in figure 1.

When 300 ml. of cellugel I were used as the initial vehicle and 1 gm. of streptomycin was added daily for ten days, the concentration of streptomycin in the pleural fluid remained constant on a high level, approximately 500 to 600 $\gamma$ per ml., for seven days and decreased slowly on the eighth or ninth day to approximately 400 $\gamma$ per ml. (figure 1).

Due to the chemical character of streptomycin, this compound could not be tested in cellugel II.

*Dihydrostreptomycin:* With a *single* intrapleural dose of 1 gm. of dihydrostreptomycin in 300 ml. of 0.9 per cent NaCl, there was a rapid fall in the concentration from an initial value of 2,200 $\gamma$ per ml. to 500 $\gamma$ per ml. after twenty-four hours and to 190 $\gamma$ per ml. after forty-eight hours (figure 2). In 300 ml. of cellugel II, a corresponding *single* dose of 1 gm. of dihydrostreptomycin caused a rapid drop in the concentration from an initial value of 2,600 $\gamma$ per ml. to 915 $\gamma$ per ml. after twenty-four hours, to 244 $\gamma$ per ml. after forty-eight hours, and to 100 $\gamma$ per ml. after seventy-two hours (figure 2). Thus, twenty-four hours after instillation the concentration of dihydrostreptomycin was higher in cellugel II than in 0.9 per cent NaCl; at the end of forty-eight hours, this difference had practically disappeared.

With a *daily* dose of 1 gm. of dihydrostreptomycin for six to ten days, the following results were seen (figure 3). With 300 ml. of 0.9 per cent NaCl as the

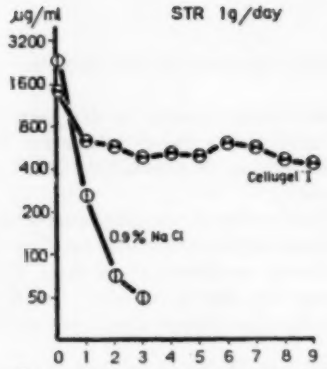


FIG. 1. Ordinate: concentration of drug in γ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: 0.9 per cent NaCl, average of three experiments; cellugel I, average of five experiments.

Dose: streptomycin, 1 gm. per day.

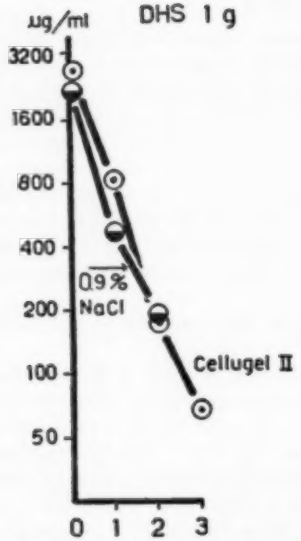


FIG. 2. Ordinate: concentration of drug in γ per ml. (log scale). Concentration obtained immediately before each instillation.

Abscissa: time in days.

Vehicle: 0.9 per cent NaCl, average of two experiments; cellugel II, average of seven experiments.

Dose: dihydrostreptomycin, 1 gm., single dose.

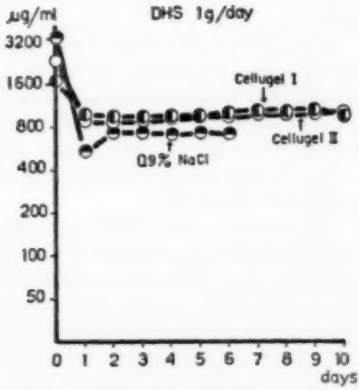


FIG. 3. Ordinate: concentration of drug in γ per ml. (log scale). Concentration obtained immediately before each instillation.

Abscissa: time in days.

Vehicle: 0.9 per cent NaCl, average of three experiments; cellugel I, average of eight experiments; cellugel II, average of five experiments.

Dose: dihydrostreptomycin, 1 gm. per day.

vehicle, the concentration in the pleural fluid varied around 700 γ per ml. With 300 ml. of cellugel I as the vehicle, a reasonably constant, somewhat higher concentration was seen, i.e., approximately 900 to 1,000 γ per ml; with 300 ml. of cellugel II as the vehicle, the concentration was practically the same, approximately 900 to 1,050 γ per ml.

*pH of pleural exudate:* One of the main objects of the investigation was to find a suitable vehicle which would produce a pH which would give optimal streptomycin effect, i.e., pH $\geqq$ 7.8.

The pH of the pleural fluid was determined three to ten times during each series, 215 times in all. The data are collected in table 3.

The pH of *0.9 per cent NaCl* was constant at approximately 6.8 to 7.0, with only minor deviations. No difference was seen between streptomycin and dihydrostreptomycin in either this vehicle or in cellugel.

In *cellugel I*, the pH showed a transient increase up to 8.0 to 8.2 immediately after the administration of the vehicle and the drug into the pleural cavity. After twenty-four hours, it was found to be approximately 7.0, and thereafter it was constant at this level during the whole period of treatment.

TABLE 1

pH IN PLEURAL FLUID WITH DIFFERENT VEHICLES

| VEHICLE | NUMBER OF EXPERIMENTS | AVERAGE | RANGE |
|---|---|---|---|
| 0.9 per cent NaCl | 6 | 6.9 | 6.8–7.1 |
| Cellugel I | 12 | 7.0 | 6.7–7.3 |
| Cellugel II | 26 | 7.9 | 7.6–8.1 |

In *cellugel II*, the pH lay constantly on a higher level, 7.8 to 8.0, during the whole ten-day period of treatment. This shows that the buffering of cellugel II with dibasic sodium phosphate was effective in maintaining a high level of pH. This pH level is optimal for the effect of streptomycin and dihydrostreptomycin.

Thus, a therapeutically optimal effect ought to be obtained with dihydrostreptomycin in cellugel II.

## DISCUSSION

The experiments related above reveal some interesting facts.

First, the rapid decrease and low concentration of streptomycin in the pleural fluid, when given with 0.9 per cent NaCl as the vehicle, despite the local application of a dose as high as 1 gm. of streptomycin per day, are striking. The amounts of streptomycin, mentioned in various reports as having been injected into the pleural cavity, usually with 0.9 per cent NaCl as the vehicle, were as a rule less than 1 gm. and often were not administered daily. Most probably, an effective concentration of streptomycin was not achieved in these cases. This might be one of the explanations of the many failures that have hitherto been reported in connection with the use of streptomycin in the treatment of tuberculous empyema.

Second, there was a considerable difference between the concentrations of streptomycin in 0.9 per cent NaCl and in cellugel I, in spite of the fact that the experimental conditions were the same in all other respects. The same difference, although not so accentuated, was seen between the concentrations of dihydrostreptomycin in 0.9 per cent NaCl, on the one hand, and in cellugel I and II, on the other hand.

Berkman, Housewright, and Henry (2) have demonstrated that, if cellophane membranes (cellulose) are placed in an aqueous solution of 50 γ of streptomycin per ml., approximately 30 per cent of the streptomycin will be adsorbed to the cellulose. If these cellophane membranes were placed afterwards in a 2 per cent NaCl solution overnight, 50 per cent of the adsorbed streptomcyin was recovered. The writers discuss the possibilities for achieving a depot effect with the aid of streptomycin bound to substances of high molecular weight for practical purposes.

The present experiments revealed a very marked difference in the concentration of streptomycin in 0.9 per cent NaCl and in cellugel I and a similar, not quite so pronounced, difference in respect to dihydrostreptomycin. The explanation of this difference might be that an adsorption of streptomycin or of dihydrostreptomycin to the large cellulose molecules had taken place and that a depot effect had arisen which kept the concentration at the higher level in cellugel I and II. With a single dose of streptomycin or dihydrostreptomycin, a higher concentration in cellugel would therefore be expected during the first few days, after which the concentration in 0.9 per cent NaCl and in cellugel would be the same. The values obtained (figure 2) coincide well with this assumption.

Third, the essential difference in pH between cellugel I and II (table 1) was apparently of no significance to the dihydrostreptomycin level (figure 3).

As may be seen from figure 1, continuous daily doses of 1 gm. of streptomycin, administered intrapleurally with cellugel I as the vehicle, produced a concentration of 500 to 600 γ per ml. No other dosages were tried because dihydrostreptomycin was found to give higher concentration with the same dose (*see* figure 6*B*).

With a single dose of 1 gm. of dihydrostreptomycin in 300 ml. of cellugel II, the concentration fell rapidly from a high initial value of 2,600 γ per ml. to 100 γ per ml. after seventy-two hours (figure 4*A*). With a daily intrapleural dose of 1 gm. of dihydrostreptomycin in the same amount of cellugel II, the concentration remained essentially constant at approximately 900 to 1,050 γ per ml; with *0.5 gm. daily*, approximately 400 to 600 γ per ml.; and with *1 gm.* once *every two days*, the concentration was approximately 200 to 300 γ per ml. after forty-eight hours (figure 4*A*). In all of these cases the values showed only slight deviation from day to day. On the other hand, with *0.5 gm.* a steadily decreasing concentration was obtained which reached an average value as low as 50 γ per ml. after ten days (figure 4*C*).

Curves of the individual experiments with dihydrostreptomycin in cellugel II in various dosages are given in figures 5 *A–D*. The variations between the individual subjects were rather slight (figures 5 *A–C*) with one exception (5 *D*).

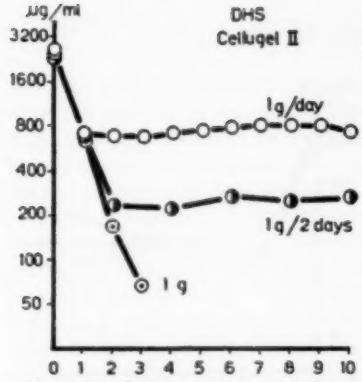


FIG. 4A. Ordinate: concentration of drug in $\gamma$ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: dihydrostreptomycin, 1 gm. per day, average of five experiments; dihydrostreptomycin, 1 gm. every two days, average of four experiments; dihydrostreptomycin, 1 gm. single dose, average of seven experiments.

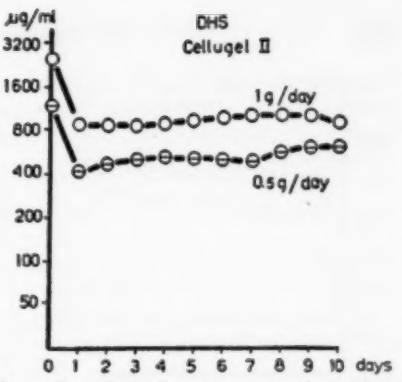


FIG. 4B. Ordinate: concentration of drug in $\gamma$ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: Dihydrostreptomycin, 1 gm. per day, average of five experiments; dihydrostreptomycin, 0.5 gm. per day, average of six experiments.

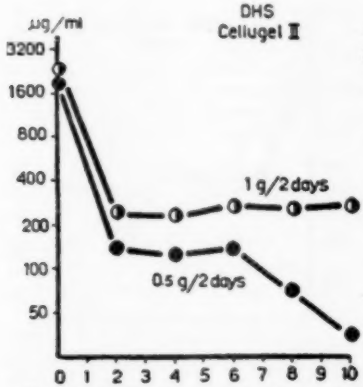


FIG. 4C. Ordinate: concentration of drug in $\gamma$ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: dihydrostreptomycin, 1 gm. every two days, average of four experiments; dihydrostreptomycin, 0.5 gm. every two days, average of five experiments.

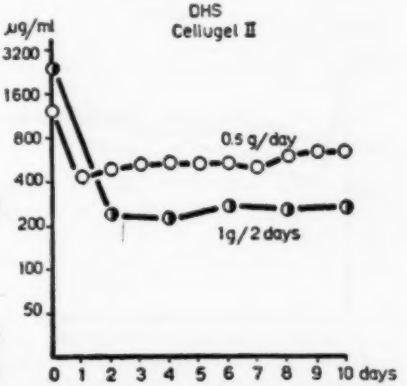


FIG. 4D. Ordinate: concentration of drug in $\gamma$ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: dihydrostreptomycin, 0.5 gm. per day, average of six experiments; dihydrostreptomycin, 1 gm. every two days, average of four experiments.

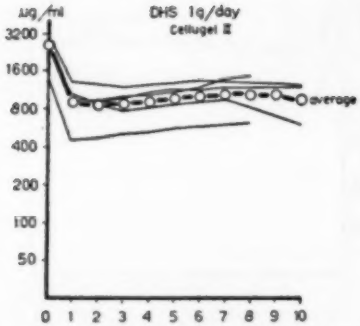


FIG. 5A. Ordinate: concentration of drug in γ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: dihydrostreptomycin, 1 gm. per day, average curve and individual curves.

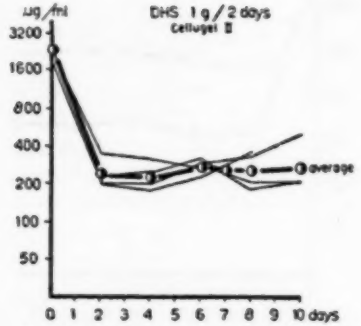


FIG. 5B. Ordinate: concentration of drug in γ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: dihydrostreptomycin, 1 gm. every two days, average curve and individual curves.

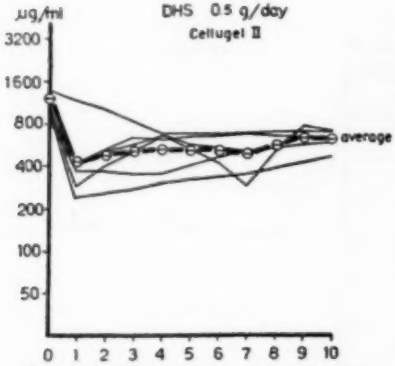


FIG. 5C. Ordinate: concentration of drug in γ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: dihydrostreptomycin, 0.5 gm. per day, average curve and individual curves.

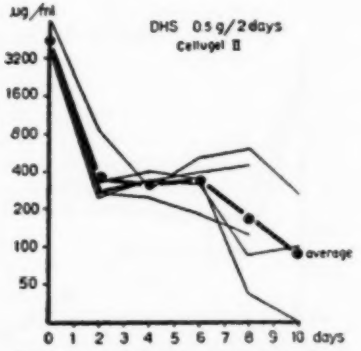


FIG. 5D. Ordinate: concentration of drug in γ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel II.

Dose: dihydrostreptomycin, 0.5 gm. every two days, average curve and individual curves.

The curves from the individual patients given 0.5 gm. of dihydrostreptomycin every second day showed that, after a lapse of one week, the concentration in some cases approached or went below 15 γ per ml. This concentration is believed to be the minimal therapeutic level for cases of this kind. The optimal concentration is surely considerably higher.

A dose of 1 gm. of dihydrostreptomycin every two days was sufficient to give a constant concentration of 200 to 300 γ per ml. during a course of treatment lasting ten days. Such concentrations are most probably therapeutically effective. A dose of 0.5 gm. every two days, on the other hand, cannot be relied upon to maintain a sufficiently high concentration for a longer period than one week. After that time the pleural fluid should be emptied and a fresh supply of the vehicle and the drug should be administered.

In 2 patients, 1 gm. of dihydrostreptomycin was given only intrapleurally in 300 ml. of cellugel II three days in succession. The concentration simultaneously present in the pleural fluid and in the blood twenty-four hours after

TABLE 2

CONCENTRATIONS OF DIHYDROSTREPTOMYCIN* IN BLOOD AND PLEURAL FLUID

| DAY | CASE 1 | | CASE 2 | |
|---|---|---|---|---|
| | Blood Concentration | Pleural Fluid Concentration | Blood Concentration | Pleural Fluid Concentration |
| 1 | 5 | 1,730 | 5-10 | 925 |
| 2 | 2 | 1,780 | 3 | 1,150 |
| 3 | 4 | 2,300 | 6 | 1,175 |

* Simultaneous concentrations of dihydrostreptomycin in the blood and in the pleural fluid twenty-four hours after each instillation (γ per ml.).

Dose: 1 gm. of dihydrostreptomycin on day 0, 1, and 2.

Vehicle: 300 ml. of cellugel II.

each application of the drug was recorded. The concentration of dihydrostreptomycin in the pleural fluid was approximately 900 to 1,700 γ per ml. and in the blood was approximately 2 to 10 γ per ml. This shows that, in these empyema cases, dihydrostreptomycin had passed from the pleural cavity into the blood, although the amount transferred was small (table 2).

As will be seen in figure 6, *A* and *B*, there was a considerable difference between the concentration of streptomycin and dihydrostreptomycin after intrapleural administration of the same amounts. The differences were more marked when the vehicle was 0.9 per cent NaCl, but were present to a certain extent also in cellugel I. In both instances, the dihydrostreptomycin concentration was higher than that of streptomycin.

The absolute amounts of streptomycin and dihydrostreptomycin in the pleural fluid at each emptying at the end of a ten-day period were calculated from the concentration of the drugs in the pleural fluid and the total amount of exudate. Only those cases in which 1 gm. of streptomycin or dihydrostreptomycin was

administered daily were included. The emptying was performed twenty-four hours after the last instillation of drug. The results are given in table 3. The tendency disclosed by these values coincided well with the information yielded

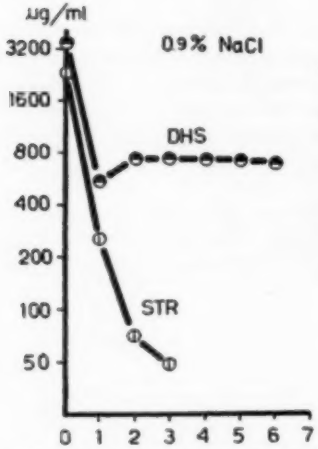


FIG. 6A. Ordinate: concentration of drug in $\gamma$ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: 0.9 per cent NaCl.

Dose: streptomycin, 1 gm. per day, average of three experiments; dihydrostreptomycin, 1 gm. per day, average of three experiments.

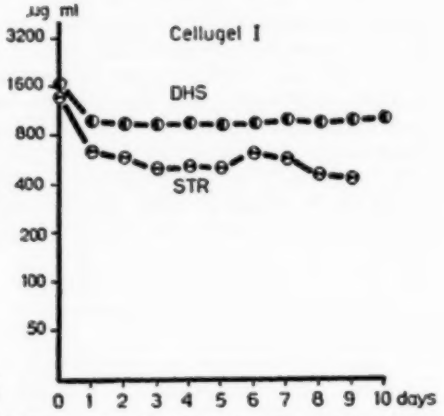


FIG. 6B. Ordinate: concentration of drug in $\gamma$ per ml. (log scale). Concentration determined immediately before each instillation.

Abscissa: time in days.

Vehicle: cellugel I.

Dose: streptomycin, 1 gm. per day, average of six experiments; dihydrostreptomycin, 1 gm. per day, average of eight experiments.

TABLE 3

AMOUNTS OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN (IN MILLIGRAMS) IN THE PLEURAL FLUID AFTER A TEN-DAY TREATMENT*

| VEHICLE | STREPTOMYCIN | DIHYDROSTREPTOMYCIN |
|---|---|---|
| 0.9 per cent NaCl | 28 (1) | 123.5 (2) |
| Cellugel I | 147 (5) | 262.0 (8) |
| Cellugel II | | 295.0 (9) |

* Dose: 1 gm. of streptomycin or dihydrostreptomycin daily. Emptying of the pleural fluid twenty-four hours after last instillation of the drug. Average values. Number of experiments in parentheses.

by the concentration curves. Thus, high dihydrostreptomycin values were found in cellugel I and II (262 and 295 mg., respectively) and lower amounts in 0.9 per cent NaCl (123 mg.). The total amounts of streptomycin, as the concen-

trations, were lower than the corresponding amounts of dihydrostreptomycin in cellugel I (147 mg.) and still lower in 0.9 per cent NaCl (28 mg.).

In these cases, fluoroscopy was used to ascertain that the emptying of the pleural cavity was practically complete. Generally, it was considered sufficient if, after rinsing, the physiological saline was clear and no more fluid was recovered than had been instilled.

If 0.9 per cent NaCl is used as the vehicle, dihydrostreptomycin ought to be the streptomycin preparation to be used exclusively for intrapleural treatment of tuberculous empyema. Even if cellugel is used as the vehicle, dihydrostreptomycin gives a higher concentration in the pleural fluid.

## SUMMARY

The intrapleural concentrations of streptomycin and dihydrostreptomycin were investigated in 44 series of experiments in 7 cases of tuberculous empyema. The drugs were administered intrapleurally, with 0.9 per cent sodium chloride (NaCl) and cellugel (a 1 per cent solution of sodium carboxymethyl cellulose) used as the vehicles. The following information was obtained.

The concentration of streptomycin in 0.9 per cent NaCl, even with a daily dose of 1 gm., remained surprisingly low; on an average, 50 to 100 $\gamma$ per ml. after forty-eight to seventy-two hours. This, in the writer's opinion, is one of the reasons for the failures of streptomycin treatment of tuberculous empyema previously reported. The doses given were apparently too small to maintain a streptomycin concentration in the pleural fluid sufficient to give a therapeutic effect.

The concentration of streptomycin was higher, 500 to 600 $\gamma$ per ml., when cellugel was used as the vehicle, and this also applies to dihydrostreptomycin in the same vehicle.

Dihydrostreptomycin showed a higher concentration in the pleural fluid than streptomycin, both when 0.9 per cent NaCl and cellugel were used as the vehicles.

With a daily intrapleural dose of 1 gm. of dihydrostreptomycin in 300 ml. of cellugel, an average concentration of 900 to 1,050 $\gamma$ per ml. was obtained; with *0.5 gm. daily*, 400 to 600 $\gamma$ per ml.; and with *1 gm. once every two days*, the concentrations were 200 to 300 $\gamma$ per ml. over a fairly long period of treatment. These concentrations are believed to be therapeutically effective. Dihydrostreptomycin, 0.5 gm. every second day in the same amount of cellugel, was not uniformly associated with a concentration above 15 to 20 $\gamma$ per ml. for longer than a week. This concentration is believed to be the minimal therapeutic concentration for cases of this kind.

If 0.9 per cent NaCl is used as the vehicle, dihydrostreptomycin ought to be the streptomycin preparation used for intrapleural treatment of tuberculous empyema. Even if cellugel is used as the vehicle, dihydrostreptomcyin is present in a higher concentration in the pleural fluid.

The pH in the pleural fluid was found to be 6.9 to 7.0, both when 0.9 per cent NaCl and unbuffered cellugel (I) were used as the vehicles.

If cellugel was buffered with dibasic sodium phosphate (cellugel II), the pH could be maintained for a considerable period at a level of 7.8 to 8.0, considered to be the optimal level for the therapeutic effect of streptomycin and dihydrostreptomycin.

The essential difference in pH between cellugel I and II was apparently of no significance in relation to the dihydrostreptomycin concentration. Cellugel II can only be used for dihydrostreptomycin.

From these findings it appears that dihydrostreptomycin given intrapleurally in cellugel II in a dose of 1 gm. every second day ought to give a therapeutically optimal effect in the intrapleural treatment of tuberculous empyema.


## SUMARIO

*La Estreptomicina y la Dihidroestreptomicina en el Tratamiento del Empiema Tuberculoso: I. Estudios Experimentales de la Concentración de la Droga Alcanzada con Varios Vehículos*

En 44 series de experimentos en 7 casos de empiema tuberculoso, investigáronse las concentraciones intrapleurales de la estreptomicina y la dihidroestreptomicina. Las drogas fueron administradas intrapleuralmente, usándose como vehículos 0.9 por ciento de cloruro de sodio (NaCl) y celugel (solución al 1 por ciento de celulosa-carboximetilo de sodio). Obtúvose la siguiente información.

La concentración de estreptomicina en 0.9 por ciento de NaCl, hasta con una dosis diaria de 1 gm., se mantuvo sorprendentemente baja: por término medio, de 50 a 100 γ por ml. al cabo de cuarenta y ocho a setenta y dos horas. Opina el A. que ésta es una de las causas de los fracasos previamente comunicados de la estreptomicinoterapia en el empiema tuberculoso. Las dosis administradas fueron aparentemente demasiado pequeñas para mantener en el líquido pleural una concentración de estreptomicina suficiente para producir efecto terapéutico.

La concentración de estreptomicina fué mayor, 500 a 600 γ por ml., cuando se usó celugel como vehículo, y esto también reza con la dihidroestreptomicina en el mismo vehículo.

La dihidroestreptomicina mostró una concentración mayor que la estreptomicina en el liquido pleural, al usar como vehículos tanto el cloruro de sodio al 0.9 por ciento cuanto el celugel.

Con una dosis intrapleural diaria de 1 gm. de dihidroestreptomicina en 300 ml. de celugel, obtúvose una concentración media de 900 a 1,050 γ por ml.; con 0.5 gm. diarios, 400 a 600 γ por ml.; y con 1 gm. una vez cada dos días, las concentraciones fueron de 200 a 300 γ por ml. durante un período bastante largo de tratamiento. Esas concentraciones parecen ser terapéuticamente eficaces. No puede contarse con que la dihidroestreptomicina, 0.5 gm., cada dos días en la misma cantidad de celugel, mantenga una concentración superior a a 15 a 20 γ por ml. por más de una semana. Esta parece ser la mínima concentración terapéutica para casos de este género.

Si se usa como vehículo cloruro de sodio al 0.9 por ciento, la dihidroesteeptomicina debe ser la preparación estreptomicínica usada exclusivamente para tratamiento intrapleural del empiema tuberculoso. Aun usando celugel como vehículo, la dihidroestreptomicina arroja una concentración mayor en el líquido pleural.

El pH del líquido pleural resultó ser de 6.9 a 7.0, al usar como vehículos tanto cloruro de sodio al 0.9 por ciento cuanto celugel sin amortiguar (I).

Si se amortiguaba el celugel con fosfato bibásico de sodio (celugel II), podía mantenerse el pH durante bastante tiempo a una cifra de 7.8 a 8.0, que pasa por

ser la óptima para el efecto terapéutico de la estreptomicina y la dihidroestreptomicina.

La diferencia fundamental del pH entre el celugel I y el II no revistió la menor importancia para la dihidroestreptomicina.

Dicha preparación, celugel II, sólo puede usarse para la dihidroestreptomicina. La dihidroestreptomicina administrada intrapleuralmente en celugel II a dosis de 1 gm. cada dos días debe, pues, producir un efecto terapéuticamente óptimo en el tratamiento intrapleural del empiema tuberculoso.


## RESUME

*Streptomycine et dihydrostreptomycine dans le traitement de l'empyème tuberculeux: I. Etudes expérimentales des concentrations de ces antibiotiques réalisées avec divers véhicules*

Les concentrations intrapleurales de streptomycine et de dihydrostreptomycine ont été étudiées au cours de 44 séries d'expérimentation dans 7 cas d'empyème tuberculeux. Ces antibiotiques étaient administrés dans la cavité pleurale, la solution de NaCl à 0,9% et le cellugel (solution à 1% de carbométhyl cellulose de sodium) étant utilisés comme véhicules. Les informations suivantes ont été obtenues.

Les concentrations de streptomycine dans la solution de NaCl à 0,9%, même à la dose quotidienne de 1 gramme, sont restées étonnamment basses, avec une moyenne de 50 à 100 $\gamma$ par ml. au bout de 48 à 72 heures. Ceci, selon l'opinion de l'auteur, est une des raisons de l'échec du traitement de l'empyème tuberculeux par la streptomycine, comme il a été rapporté précédemment. Les doses données étaient apparemment trop faibles pour maintenir dans le liquide pleural une concentration de streptomycine suffisante pour obtenir une action thérapeutique.

Les concentrations de streptomycine étaient plus élevées, 500 à 600 $\gamma$ par ml. quand le cellugel était utilisé comme véhicule; ceci est également valable pour la dihydrostreptomycine dans ce même véhicule.

La concentration de la dihydrostreptomycine dans le liquide pleural est plus élevée que celle de la streptomycine quand l'une ou l'autre ont pour véhicule la solution de NaCl à 0,9% ou le cellugel.

Avec une dose quotidienne de 1 g. de dihydrostreptomycine dans 300 ml. de cellugel, une concentration moyenne de 900 à 1.050 $\gamma$ par ml. a été obtenue; avec 0,5 g. par jour, 400 à 600 $\gamma$ par ml.; avec 1 g. une fois tous les deux jours les concentrations étaient de 200 à 300 $\gamma$ par ml.; ceci, pendant une période de traitement assez prolongé. On estime que ces concentrations possèdent une valeur thérapeutique. La dihydrostreptomycine, à la dose de 0,5 g. tous les deux jours dans la même quantité de cellugel, ne permet pas de compter sur le maintien d'une concentration dépassant 15 ou 20 $\gamma$ par ml. pendant plus d'une semaine. Cette limite est considérée comme la concentration thérapeutique minima pour les cas de cette sorte.

Si la solution de chlorure de sodium à 0,9% est utilisée comme véhicule, la dihydrostreptomycine devrait être la préparation de streptomycine exclusivement utilisée pour le traitement intra-pleural de l'empyème tuberculeux. Même dans le cas où le cellugel est utilisé comme véhicule, la dihydrostreptomycine procure une concentration plus élevée dans le liquide pleural.

La valeur du pH du liquide pleural était de 6,9 à 7, aussi bien quand la solution de chlorure de sodium à 0,9% et le cellugel non tamponné (cellugel I) ont été utilisés comme véhicules.

Lorsque le cellugel était tamponné avec le phosphate de sodium dibasique (cellugel II), le pH pouvait être maintenu pendant une période considérable à une valeur de 7,8 à 8, considérée comme étant le pH le plus favorable à l'effet thérapeutique de la streptomycine et de la dihydrostreptomycine.

La différence essentielle dans le pH, entre le cellugel I et II était, apparemment sans importance quant à la concentration de la dihydrostreptomycine.

Cette préparation, le cellugel II, ne peut être employée que pour la dihydrostreptomycine.

Donc, la dihydrostreptomycine intrapleurale dans le cellugel II, à la dose de 1 g. tous les deux jours, devrait procurer un effet thérapeutique optimum dans le traitement intrapleural de l'empyème tuberculeux.

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# STREPTOMYCIN AND DIHYDROSTREPTOMYCIN IN THE TREATMENT OF TUBERCULOUS EMPYEMA[1]

## II. Studies with Cellugel as Vehicle

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### INTRODUCTION

Treatment of tuberculous empyema with streptomycin, applied locally, has in general not come up to expectations (1, 2, 5, 6, 9, 10, 11, 12, 13, 14, 15, 18, 22, 24, 28, 29). Hinshaw and collaborators believed this to be due, in part, to the acid reaction of the pus (the effect of streptomycin is greatly reduced in an environment of this kind) as well as to granulomatous changes in the walls and to hydrolytic protein products in the pus which hamper the action of streptomycin. In Riggins and Hinshaw's book on streptomycin and dihydrostreptomycin, published in 1949 (12), the following statement appears: "The chances for the successful control of tuberculous empyema would appear to be exceedingly remote." This pessimistic view, however, is not shared by all investigators. Several writers report that they have achieved a cure in some cases by means of repeated rinsings with 0.9 per cent sodium chloride (NaCl) and intrapleural administration of streptomycin, often combined with intramuscular streptomycin (7, 8, 16, 17, 19, 20, 26). The best results seem to be achieved in extrapleural empyema and in acute empyema. French and Italian investigators, in particular, have attempted to alkalinize the pus of empyema in order to improve the chances for success (5, 21, 25). The clinical results seem to have been fairly good. Christensen (8) used alkalinization with success in five of his empyema patients, two of whom had bronchopleural fistulas. However, he advised against the practice of keeping the pH at the 8.0 level, as he considered this would be injurious for the tissues, but his reasons for adopting this view are not stated. Padovani and Jotti (21) maintained that alkalinization promotes the regenerative processes and ought, therefore, to be used as much as possible. Posternak (23) treated cases of empyema with streptomycin and dihydrostreptomycin in a polyvinyl alcohol solution buffered to pH 7.5 with borax solution and succeeded in curing nine patients. Guinea pig tests, carried out afterwards with the exudate, also proved to be negative. The results were also good in empyema of long standing.

The present writer has previously shown (27) that, if cellugel (a 1 per cent solution of sodium carboxymethyl cellulose) is used as the vehicle and 1 gm. of dihydrostreptomycin is added to it once every two days, a fully satisfactory therapeutic concentration of dihydrostreptomycin is obtained. If this cellugel is buffered with dibasic sodium phosphate, the pH can be maintained at the level ensuring an optimal streptomycin effect (pH $\geqq$ 7.8). The concentrations of streptomycin and dihydrostreptomycin were higher in cellugel than in 0.9 per

[1] From Kolmården Sanatorium, Sweden.

cent NaCl, in spite of the fact that the experimental conditions were the same in all other respects.

## METHODS AND PROCEDURE

*Test subjects:* Seven cases of empyema, including 2 of the extrapleural type, were studied. In addition, a case of pleural empyema with a bronchopleural fistula was treated, but this case will be discussed separately.

*Vehicles:*

Cellugel I

| | |
|---|---|
| Sodium carboxymethyl cellulose | 10.0 gm. |
| Sodium chloride | 8.0 gm. |
| Nipagin[2] | 1.0 gm. |
| Sterile water | 1,000.0 ml. |

1 Normal sodium hydroxide to make pH = 9.5. Twenty-four hours after the application intrapleurally of the vehicle, pH was approximately 7.0.

Cellugel II

| | |
|---|---|
| Sodium carboxymethyl cellulose | 10.0 gm. |
| Disodium phosphate | 16.7 gm. |
| Nipagin[2] | 1.0 gm. |
| Sterile water | 1,000.0 ml. |

pH = 8.15 (pH = 7.8 to 8.0 for a ten-day period of treatment)

*Preparations:*

Streptomycin: The preparation used was a complex salt of streptomycintrichloride and calcium chloride, containing 0.730 gm. streptomycin base per gm.

Dihydrostreptomycin: The preparation used was bisdihydrostreptomycintrisulfate, containing 0.780 gm. base per gm.

Nipagin is added in order to better conserve the solution.

### *Procedure*

With the patient on his back, and after local anesthesia had been produced, a puncture was made with a long, coarse, specially constructed needle into the second intercostal space. The head-end of the underlying support was then lowered slightly. The emptying was done with a water pump or a hypodermic syringe. In this way, the exudate can be emptied rapidly and completely, controlled by fluoroscopy. The risk of secondary fistula formation is extremely slight, as the area of the puncture hardly comes into contact with the empyema fluid at all. With this technique, a parietal fistula occurred in only one case (Case 8 with a bronchopleural fistula) and then only after many emptyings. The position in question is comfortable for both the patient and the operator, and the fainting attacks liable to occur in connection with other methods are avoided.

After a complete withdrawal of the empyema fluid and rinsing with 0.9 per cent NaCl until a clear solution was obtained, 300 ml. of cellugel (I or II) were applied, to which 1.0 gm. of streptomycin or varying amounts of dihydrostreptomycin, 0.5 to 1.0 gm., were added. Then streptomycin (10 gm.) was added daily, or dihydrostreptomycin (0.5 to 1.0 gm.) daily or once every two days, to the pleural fluid for up to ten days. The cellugel was changed every ten days.

Streptomycin and dihydrostreptomycin were only administered locally into the pleural fluid in this study.

[2] Nipagin is methylparabenzoate (methylparaben in the American Pharmacopea).

## RESULTS

*Treatment with streptomycin or dihydrostreptomycin in a nonalkaline cellugel solution (I), pH = 7.0:* Four empyema cases (Cases 1, 2, 3, 4), three of pleural empyema and one of extrapleural empyema, were treated in this way. Tubercle bacilli of the human type had been demonstrated in the exudates from all of the cases. One of the patients with pleural empyema and the patient with ex-

TABLE 1

INTRAPLEURAL TREATMENT OF TUBERCULOUS EMPYEMA WITH STREPTOMYCIN OR DIHYDROSTREPTOMYCIN IN NONALKALINE (C I) OR ALKALINE (C II) CELLUGEL SOLUTION

| CASE NUMBER | ONSET OF EMPYEMA | INTRAPLEURAL TREATMENT | | | TUBERCLE BACILLI IN EXUDATE | | CLINICAL RESULT |
|---|---|---|---|---|---|---|---|
| | | Days | Drug | Vehicle | Before | After | |
| 1, B. R., male | 1949; extrapleural pneumothorax | 19<br>21 | SM<br>DHSM | C I<br>C I | Cult. +<br>G.p. + | Cult. –<br>G.p. – | Cured; no exudate |
| 2, A. K., male | 1947 | 24 | SM | C I | Cult. +<br>G.p. + | Cult. –<br>G.p. – | Cured; slight exudate |
| 3, I. G., female | 1949 | 31<br>121 | SM<br>DHSM | C I<br>C II | +<br>on smear | Cult. –<br>G.p. – | Cured; no exudate |
| 4, N. E., male | 1949 | 15<br>21<br>62 | SM<br>DHSM<br>DHSM | C I<br>C I<br>C II | Cult. +<br>G.p. + | Cult. –<br>G.p. – | Cured; no exudate |
| 5, V. J., female | 1950 | 54 | DHSM | C II | Cult. +<br>G.p. + | Cult. –<br>G.p. – | Cured; no exudate |
| 6, G. P., male | 1950; extrapleural pneumothorax | 66 | DHSM | C II | Cult. +<br>G.p. +<br>Bovine tuberculosis | Cult. –<br>G.p. + | Cured; no exudate |
| 7, S. A., male | 1947 | 33 | DHSM | C II | Cult. +<br>G.p. + | Cult. –<br>G.p. + | Cured; slight exudate |
| 8, N. A., male | 1949 | 147 | DHSM | C II | +<br>on smear | Cult. +<br>G.p. + | No cure |

SM = streptomycin.
DHSM = dihydrostreptomycin.
Cult. = cultivation on Löwenstein medium.
G.p. = inoculation on guinea pig.
C I = cellugel I.
C II = cellugel II.

trapleural empyema recovered rapidly (Cases 1 and 2); the other 2 showed only slight improvement. In the 2 cured patients, both cultures and guinea pig inoculations, using pleural washings, were negative for *M. tuberculosis* at the termination of the treatment. One year after the end of treatment, in the patient with the extrapleural empyema no exudate has reappeared; and, in the other patient who was cured, a small amount of clear exudate developed after six months but has since shown no signs of increasing. In the 2 patients who were not cured, treatment was continued in accordance with the principles applied in group 2, and these 2 cases are included in that group (table 1).

*Treatment with dihydrostreptomycin in cellugel (II) with a pH giving the optimal effect of streptomycin (pH in cellugel = 8.15):* The series consisted of 5 cases (Cases 3, 4, 5, 6, 7), including 4 of pleural empyema with tubercle bacilli of human type and one of extrapleural empyema with bovine tubercle bacilli in both the empyema fluid and the sputum (Case 6). In all of these cases, a clear exudate was obtained after a course of treatment of varied duration. After the termination of the treatment, cultures and guinea pig inoculations with the exudate were negative for *M. tuberculosis* in 3 cases, while in 2 cases (Cases 6 and 7) the guinea pig inoculations alone were positive. In one of the last-named 2 cases, the pleural cavity is now dry; in the other, there is still a small amount of thin clear exudate which contains tubercle bacilli as shown by guinea pig inoculation. In the remaining cases there is now no exudate. There were no recurrences after an observation time of six to nine months (table 1).

In one case (Case 8) there was pleural emypema with a bronchopleural fistula. In this patient, after a long course of treatment (146 days) in accordance with the principles mentioned above, the only result achieved was that the pus became less thick. The fistula was still open. In this case, the concentration of dihydrostreptomycin was strikingly high despite the constant expectoration of empyema fluid, and the pH in the cellugel was approximately or well over 8.0. Towards the end of the treatment period, the patient was given a couple of courses of therapy with 0.9 per cent NaCl as the vehicle; and, during these periods also, the pH was remarkably high (7.5). This finding is interpreted as indicating that considerable alkalinization of the wall had taken place owing to the protracted treatment with an alkaline gel.

## DISCUSSION

Of 4 cases of tuberculous empyema treated with streptomycin or dihydrostreptomycin in a nonalkaline cellugel solution, complete recovery was achieved in 2 cases. Of 5 cases treated with dihydrostreptomycin in a cellugel solution, the level of pH in it being optimal for the effect of streptomycin and dihydrostreptomycin, all were cured. No injurious effects were observed as the result of the alkalinization in these cases.

Increased scarring as a result of the cellugel treatment was not observed in any of these cases. On the contrary, even a nearly normal diaphragmatic function was seen after the treatment in some cases, for instance in Case 5, making a secondary surgical treatment unnecessary.

The distinctly better results obtained with the latter method of treatment suggest strongly that continuous alkalinization of the empyema fluid is of considerable significance to the healing process during treatment with streptomycin and dihydrostreptomycin. Thus, also in old empyemas, it was possible to obtain a cure with dihydrostreptomycin in an alkaline cellugel solution.

Of course, the above-mentioned treatment of tuberculous empyema with dihydrostreptomycin in a 1 per cent cellugel solution is not always sufficient because in old empyemas the thickening of the visceral pleura prevents the expansion of the lung, thus requiring a secondary surgical treatment in order to close the pleural space. According to the writer's experience in acute empyemas, on the other hand, the therapy in most cases gives a very good final result with astonishingly good lung function.

The treatment was unsuccessful in one case, probably owing to the presence of a persistent pleurobronchial fistula. It is questionable whether the alkalinization of the empyema fluid has anything to do with the persistency of the fistula. In one of Christensen's (8) cases in which the pH was maintained at a high level (8.5), a pleurobronchial fistula was present and closed during the treatment. Padovani and Jotti (21) considered that alkalinization on the contrary promoted regeneration.

In the decision as to when treatment should be terminated, there are two criteria which must be followed. One is that the exudate, when emptied, must be grossly clear at least once after a course of treatment of ten days. The other essential is that the absorption tendency must dominate; in other words, when the emptyings are carried out, the recovery must be less than the amount that has been applied. In the 7 cases of empyema in which a cure was effected, the time which elapsed before these conditions were fulfilled varied from ten to ninety days, the average being thirty-six days.

## SUMMARY

Four patients with empyema were treated with streptomycin or dihydrostreptomycin in a nonalkaline cellugel solution (a 1 per cent solution of sodium carboxymethyl cellulose). Two of these were cured.

Five patients with empyema (one caused by bovine tubercle bacilli) were treated with dihydrostreptomycin (0.5 to 1.0 gm. daily or every second day) in an alkaline cellugel solution (pH = 7.8). All 5 of these patients were cured. No injurious effects were noted as the result of the alkalinization. No increased scarring as a result of the cellugel treatment was observed. In another case with a persistent pleurobronchial fistula, the treatment was unsuccessful.

Continuous alkalinization of the empyema fluid seems to be of considerable significance to the healing process during treatment with streptomycin or dihydrostreptomycin, the effect of the drugs being improved in this way. Even in old empyemas, it was possible to obtain a cure with dihydrostreptomycin in an alkaline cellugel solution. Nevertheless, in old empyemas, a secondary surgical treatment is often required in order to close the pleural space; in acute empyemas, this is not usually necessary.

SUMARIO

*La Estreptomicina y la Dihidroestreptomicina en el Tratamiento del Empiema Tuberculoso: II. Estudios con Celugel como Vehículo*

Cuatro casos de empiema fueron tratados con estreptomicina o dihidroestreptomicina vehiculizada en una solución no alcalina de celugel (solución al 1 por ciento de celulosa-carboximetilo de sodio). Dos de ellos sanaron.

Cinco casos de empiema (uno con tuberculosis bovina) fueron tratados con dihidroestreptomicina (0.5 a 1.0 gm. diarios o cada dos días) en una solución alcalina de celugel (pH de 7.8). Todos esos enfermos se curaron. No se notó ningún efecto nocivo a consecuencia de la alcalinización. En un caso con una persistente fístula pleurobronquial, el tratamiento no dió resultado.

La alcalinización continua del líquido empiematoso parece revestir considerable importancia en el proceso curativo durante el tratamiento con estreptomicina o dihidroestreptomicina, realzándose de ese modo el efecto de los antibióticos.

Fué, pues, así posible obtener también, en los empiemas antiguos la curación con la dihidroestreptomicina disuelta en una solución alcalina de celugel.

RESUME

*Streptomycine et dihydrostreptomycine dans le traitement de l'empyème tuberculeux: II. Etudes avec le cellugel comme véhicule*

Quatre cas d'empyème ont été traités par la streptomycine ou la dihydrostreptomycine dans une solution non alcaline de cellugel (solution à 1% de carbométhylcellulose de sodium). Deux de ces cas furent guéris.

Cinq cas d'empyème (un par tuberculose bovine) furent traités avec de la dihydrostreptomycine (0,5 à 1 g. par jour, ou tous les deux jours) dans une solution alcaline de cellugel (pH 7,8). Tous ces malades furent guéris. Aucune réaction nocive liée à l'alcalinisation n'a été observée. Dans un cas où il y existait une fistule pleuro-bronchique persistante, le résultat du traitement a été négatif.

L'alcalinisation continue du liquide d'empyème parait avoir une importance considérable pour le processus de cicatrisation au cours du traitement par la streptomycine ou la dihydrostreptomycine, l'action des antibiotiques se trouvant améliorée par cette méthode. C'est ainsi que la guérison a pu être obtenue, même dans des empyèmes anciens, avec la dihydrostreptomycine dans une solution alcaline de cellugel.

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# A METHOD FOR STANDARDIZATION OF TUBERCULIN PREPARATIONS BY INTRACUTANEOUS REACTIONS IN HUMANS[1]

## Comparison of Two Purified Tuberculins

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### INTRODUCTION

Tuberculin is a product of tubercle bacilli. It contains one or several substances which produce biologically characteristic actions in hosts infected with tubercle bacilli. Highly active substances have been isolated from the crude extract of cultures of tubercle bacilli, but the chemical constitution of the active principles in tuberculin is still unknown, and no chemical assay has yet been developed.

With the extensive use of tuberculin for diagnostic work, for selecting individuals for BCG vaccination, and for research on tuberculosis, it is highly desirable to have a satisfactory way of estimating the amount of active substances contained in various preparations. Several methods, usually denoted as methods of standardization, based on some biologic action have been used to relate the strength of various preparations to one product arbitrarily accepted as a standard.

A requirement for any standardization is a biologic reaction which has a quantitative relationship to the amount of active substance used. This relationship can be expressed graphically as a "dose-response curve" by plotting various doses as abscissas and the corresponding responses as ordinates in a rectangular coordinate system. The efficiency of a method used for comparing two products will depend to some extent on the steepness of this curve. The greater the increase in response for a given increase in dose, the more efficient is the particular method of standardization. Determination of the dose-response curve, therefore, is essential for evaluating the utility of the method.

Doses from two preparations, e.g., the dose $d_1$ from an unknown preparation and $d_0$ from the standard, are said to be equivalent if they contain the same quantity of active substance. The inverse ratio between these doses, i.e., $d_0/d_1$, is called the ratio of potency of the preparations.

Equivalent doses must produce reactions of equal average size in the same test subjects. On the other hand, the assumption that equal mean responses indicate equivalent doses and that a ratio of potency can therefore be determined is correct only if the preparations are *qualitatively* equal. This means that the *same* substance is responsible for the biologic activities of the two preparations, or that one of the preparations can be regarded as merely a dilution of the other. In such a case the same ratio of potency should be found, regardless of the nature of the test subject or of the type of reaction used for comparison. More-

[1] From the Tuberculosis Research Office, World Health Organization, Copenhagen, Denmark.

over, every multiple of two equivalent doses should also be equivalent; that is, the same ratio of potency should be found with either large or small doses.

Not infrequently, different ratios of potency are obtained with different test subjects or methods, indicating that the preparations contain more than one active component. The relative activity of these components for different species may vary, and each of them may be particularly responsible for a certain kind of reaction. If, in addition, the active components are not present in the same proportion in two preparations, the result of a comparison will vary according to test subjects or method used.

Among the biologic properties of tuberculin, the production of a local skin reaction as well as the shock effect have been used for standardization in tuberculous guinea pigs. In some instances standardization is also carried out on human subjects by means of the skin reaction. Nevertheless, the experimental design in most of these methods has serious shortcomings. In addition to the element of personal bias in interpretation of results when the various test doses are placed on the arm according to a fixed pattern, the number of cases for testing is usually insufficient and the subjects selected are often so highly sensitive to low doses of tuberculin that they cannot be considered representative of the persons on whom the tuberculin will later be used.

It has been maintained that "skin effect" and "shock effect" are due to different components of tuberculin (1). Since the former is utilized in routine examinations and studies of human beings, it is appropriate to standardize according to the skin effect, i.e., to disregard the shock component when a ratio of potency is determined.

Even in a comparison limited to the skin effect in humans, qualitative differences between two preparations still must be considered. Human populations may be sensitized by different, although related, antigens. In this connection, it is of interest that animal work has shown that tuberculins derived from different strains of tubercle bacilli (human, bovine, avian, BCG) are qualitatively different. Each type of tuberculin is relatively favored by a test group sensitized by the corresponding (homologous) strain, but in most cases some reaction is also produced in groups sensitized by other (heterologous) strains (2, 3, 4, 5). In human populations, however, it is rarely possible to obtain direct information about the antigens responsible for sensitivity except for artificial BCG vaccination. In addition to the known types of tubercle bacilli, there may be other organisms which can induce some degree of sensitivity to tuberculin or perhaps only to a certain component of the tuberculin. Preparations containing a high proportion of this component will then be particularly potent in persons sensitized by the corresponding antigen. In fact, demonstration of anomalous sensitivity in response to a certain product may even indicate that the population in question must be sensitized by other organisms in addition to tubercle bacilli. The significance of such findings will be brought out in connection with a subsequent paper from the Tuberculosis Research Office.

Qualitative differences between preparations might appear, even if all test subjects have been sensitized by the same antigen. The subjects usually differ

in *degree* of sensitivity, and it may be impossible to find two doses which are equivalent on every level of sensitivity. The relationship between two such tuberculins could not be expressed by a single ratio of potency, and the preparations would therefore be qualitatively different.

Finally, there is the possibility that a ratio of potency determined for small doses is not valid for large doses of two preparations, or that multiples of equivalent doses are not always equivalent. This also indicates that the preparations must be considered to be qualitatively different.

Determination of whether two preparations are qualitatively different is an essential part of the comparison of tuberculins. Serious problems are not caused by a mere quantitative difference, since equivalence can be effected by simply adjusting the dose. If qualitative differences exist, however, one preparation cannot be substituted for another by any adjustment of the dose, even though equal mean responses may be produced under certain conditions.

The present report describes the experimental design, the analysis, and the results of a comparison of two purified tuberculins, *PPD-S* and *RT XIX-XX-XXI*. PPD-S was produced in 1940 as the international standard PPD under the supervision of Dr. Florence Seibert. It has been used in large quantities during the past eleven years in the United States for routine purposes as well as for extensive research studies. Smaller quantities have been sent to several countries of Europe, Central and South America. RT XIX-XX-XXI is a mixture of three lots prepared in 1945 to 1946 at Statens Seruminstitut in Copenhagen, Denmark. It was used for the International Tuberculosis Campaign from April, 1948 to February, 1950 on approximately six or seven million persons in Poland, Czechoslovakia, Yugoslavia, Hungary, and Greece. It has also been used for investigations of tuberculin sensitivity and post-vaccination allergy conducted by the Tuberculosis Research Office in Copenhagen.

## MATERIALS AND METHODS

### *Preparations of the Purified Tuberculins*

Dr. Florence Seibert has kindly provided the following summary of the preparation of PPD-S:

> Human-type tubercle bacilli, strain DT, were grown on Long's synthetic medium for eight to ten weeks at 37.5°C. The cultures were heated in the Arnold sterilizer for three hours and then filtered first through a Buchner funnel and then through a Mandler candle, at a temperature of 4° to 5°C. At this temperature bacteria-free filtrate was concentrated by means of ultrafiltration and washed with weak phosphate buffer of pH 7.3, containing 0.8 per cent toluol. The final concentration was 0.7 to 1.3 per cent protein. An equivalent volume of neutralized saturated ammonium sulfate solution was added to the solution and the precipitation repeated seven more times. The final precipitate was re-dissolved, clarified by filtration through a Mandler filter, washed free of ammonium sulfate on the ultrafilter, again filtered through the Mandler filter to insure sterility, and lyophilized in quantities containing 10 or 50 mg. of protein, based on nitrogen analyses. The vials were sealed under vacuum. The final product contained 1.2 per cent nucleic

acid and 5.9 per cent carbohydrate, and was highly potent in the skin test on tuberculous patients.

For further details *see* SEIBERT, F. B., AND GLENN, J. T., Am. Rev. Tuberc., 1941, *44*, 9.

The preparation RT is prepared in the following way:

Human-type tubercle bacilli are grown on Sauton's medium for five weeks at 37°C., killed by sterilizing for one hour in flowing steam at 1.2 atmosphere, and filtered first through a double layer of filter paper and then through a bacteria-proof filter (Berkefeld filter). The filtrate is concentrated to one-twentieth of the volume and washed in distilled water by ultrafiltration through a 7 per cent collodium membrane. The tuberculin-active substances are precipitated from the concentrate by addition of trichloracetic acid (2 gm. per 100 ml. of concentrate). The resulting sediment is washed with 2 per cent trichloracetic acid solution and, after washing and dehydration with water-free ether, the purified tuberculin is obtained as an amorphous powder.

For further details *see* JENSEN, K. A., BINDSLEV, G., MÖLLER, S., HANSEN, A., AND LIND, P., Tubercle, 1938, *19*, 443; and LIND, P., Acta tuberc. Scandinav., 1947, *21*, 137.

Both purified tuberculins used in this study were diluted to the specified strengths in a buffer with pH = 7.38 and composed as follows:

| | |
|---|---|
| m/15 disodium acid phosphate | 64 ml. |
| m/15 potassium dihydrogen phosphate | 16 ml. |
| 2.4 per cent sodium chloride with 0.05 per cent chinosol | 20 ml. |
| Total | 100 ml. |

### *General Design of the Studies*

The present investigation comprises a series of studies, designed partly for a determination of the dose-response curve for RT tuberculins, partly for a comparison of PPD-S and RT XIX-XX-XXI. The following general principles apply to these studies.

The measured size of induration to the intradermal Mantoux test was used as a quantitative estimate of the biologic response to a given dose of tuberculin. Two tests, symmetrically placed on the dorsal surfaces of the forearms, were given to each person. A fixed control dose was used as one of the tests, given alternately on the left and right arms. The test on the other arm was one of three, or in some studies four, variable doses. To obtain comparable groups the variable doses were given by rotation to the subjects in the order they met for examination.

Within each study all tests were given by the same tester, who carefully injected 0.1 ml. of dilution intradermally from non-leaking syringes. The syringes containing various doses were clearly marked to enable the assistant to check and record the application of doses and preparations in accordance with the plan.

Readings were made after seventy-two hours and always by the same reader throughout each study. Transverse diameters of erythema and induration were measured as accurately as possible, first on the left and then on the right arm, and results were dictated to the secretary in this order. The reader did not know the identity of the tests being read.

This design of the investigation was used in order to avoid influence of systematic errors or personal bias on the results. It is essential that the tests for various preparations and doses are distributed at random in the total number of subjects and that all observations are made without knowledge of the design of this distribution.

## OBSERVATIONS

### *Preliminary Dose-Response Studies*

Three preliminary studies were performed prior to comparing the two preparations in order to determine the dose-response relationship for the RT tuberculins.[2]

*Study I* and *Study III* were conducted in October, 1950, and June, 1951, in two rural areas of Denmark in connection with yearly re-examinations of groups comprising 375 and 791 BCG-vaccinated school children, respectively. Both groups were evenly divided as to sex, all children were within the age span of 7 to 16 years, and most of them had been vaccinated in 1948. In *Study I*, three different doses, 1, 5, and 10 tuberculin units (TU), were compared with 5 TU as the control dose. In *Study III*, four variable doses were compared, 2.5, 5, 7.5, and 10 TU, with 10 TU as the control dose. In the latter study all reactions were read independently by two readers. The tuberculin used in these studies was RT XIX-XX-XXI from Statens Seruminstitut in Copenhagen.[3]

TABLE 1

MEAN SIZES OF INDURATION TO MANTOUX TESTS WITH VARIOUS DOSES OF RT IN BCG-VACCINATED SCHOOL CHILDREN (STUDIES I AND III) AND IN PATIENTS FROM A PSYCHIATRIC HOSPITAL (STUDY II)

| SUB-GROUP TESTED WITH SPECIFIC DOSE | NUMBER OF PERSONS TESTED IN: | | | MEAN SIZE OF INDURATION TO THE SPECIFIC DOSE (RESULT OF CONTROL DOSE GIVEN IN PARENTHESES) | | | |
|---|---|---|---|---|---|---|---|
| | Study I | Study II | Study III | Study I (Control Dose, 5 TU) | Study II (Control Dose, 5 TU) | Study III, Reader I (Control Dose, 10 TU) | Study III, Reader II (Control Dose, 10 TU) |
| 1.0 TU | 120 | | | 4.38 (7.18) | | | |
| 1.25 TU | | 313 | | | 8.86 (11.80) | | |
| 2.5 TU | | 295 | 197 | | 9.97 (11.80) | 7.54 (14.10) | 7.87 (14.46) |
| 5.0 TU | 124 | 297 | 200 | 7.48 (7.21) | 11.11 (11.71) | 9.59 (13.62) | 10.06 (13.82) |
| 7.5 TU | | | 198 | | | 11.86 (14.11) | 12.04 (14.21) |
| 10.0 TU | 131 | 299 | 196 | 10.33 (6.61) | 16.94 (12.30) | 12.62 (13.25) | 12.88 (13.46) |

*Study II* was made on 1,204 mentally ill patients (564 males and 640 females) who ranged in age from 15 to 95 years. Although reliable information on previous BCG vaccination could not be obtained, apparently only a few had been vaccinated. Four variable doses, 1.25, 2.5, 5, and 10 TU, were used, with 5 TU as the control dose, all from batch RT XXII from Statens Seruminstitut.[4]

The subjects in each study have been sub-grouped according to the variable dose which they received as one of the tests. Within each sub-group the variable or specific dose and the fixed control dose were used equally often on the left and right arms.

[2] A detailed account of these studies will be given in a subsequent paper from the Tuberculosis Research Office by Dr. Johs. Guld.

[3] One tuberculin unit of batch RT XIX-XX-XXI contains 1/50,000 mg. of the dry PPD substance.

[4] One tuberculin unit of batch RT XXII contains 1/75,000 mg. of the dry PPD substance.

A summary of the data is presented in table 1, showing the number of persons tested together with the mean size of induration for specific dose and control dose in each sub-group. It appears that the mean size of induration to the specific dose in all studies shows a steady increase with increasing dose, while the differences in response to the control dose are insignificant and unsystematic.

A better expression for the relationship between dose and mean response may be obtained by taking the difference between mean responses for specific and

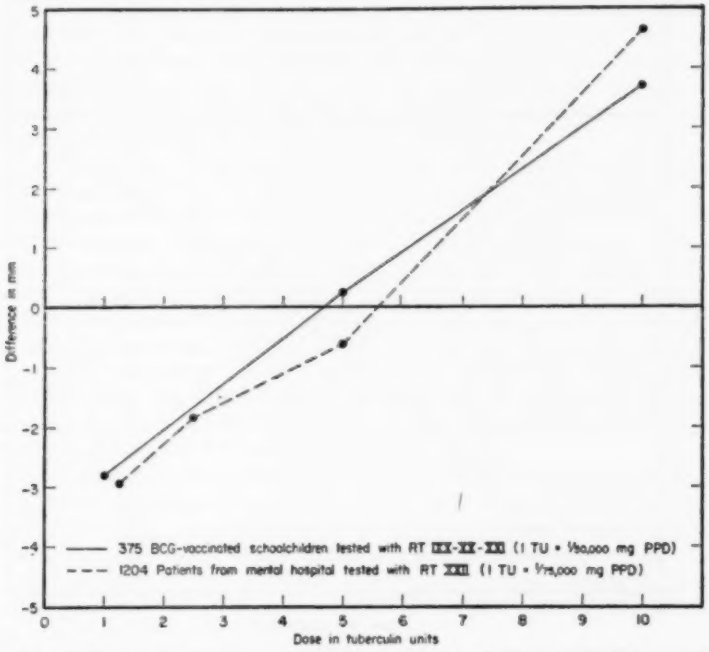


FIG. 1. Differences between mean sizes of induration to Mantoux tests with a specified dose and with 5 TU of the same purified tuberculin.

control doses. Graphic presentations of this relationship are given in figure 1 for *Studies I* and *II* (both having 5 TU as control dose) and in figure 2 for *Study III* (in which the control dose was 10 TU). The doses are plotted along the abscissa, while the ordinate shows the mean response to the specific dose as a deviation from the control in the same sub-group.

It may be seen that the relationship between dose of tuberculin and mean size of induration is approximately linear over this limited range, with an increase of approximately 0.7 to 0.9 mm. in response for each unit of tuberculin. The dose has essentially no effect in subjects with a low degree of sensitivity

(0 to 3 mm. induration to 5 TU). Among more sensitive subjects (more than 5 mm. induration to 5 TU usually classified as "positive" reactors), an increase of 1 TU results on an average in an increase of 0.9 to 1.1 mm. in size of induration.[5] Accordingly, an increase of approximately $\frac{p}{100} \times 1.0$ mm. per TU should be expected in a group comprised of $p$ per cent "reactors" and $(100\text{-}p)$ per cent "non-reactors." This does not imply that the linear relationship shown in the

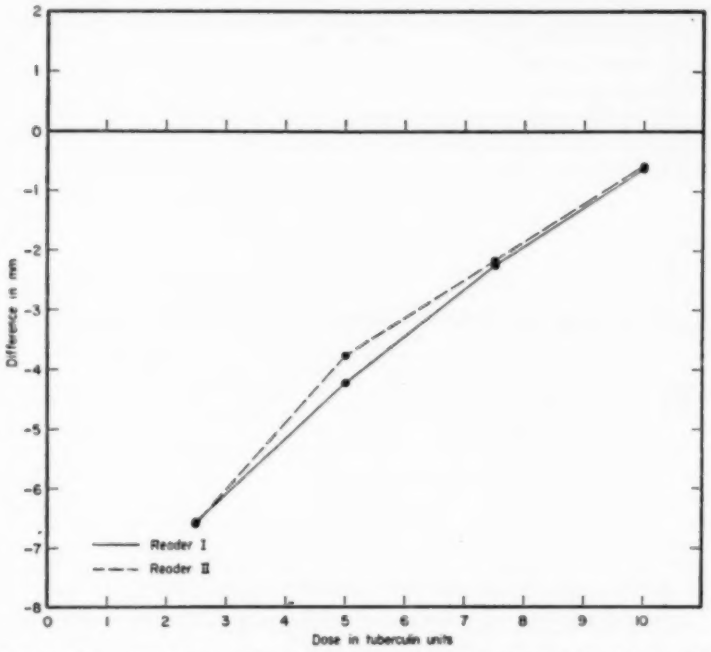


FIG. 2. Differences between mean sizes of induration to Mantoux tests with a specified dose and with 10 TU of RT XIX-XX-XXI (1 TU = 1/50,000 mg. of PPD) among 791 BCG-vaccinated school children.

figures expresses a true dose-response law, but only that for practical purposes it is a satisfactory approximation within a short range of dosage. Over a broader range the curve must deviate appreciably from a straight line if an arithmetic scale of doses is used. A logarithmic scale, however, is less satisfactory for the data in the range used here.

[5] In the subsequent paper (Johs. Guld), details and information on how this relationship was studied for "different levels of sensitivity" will be presented. The problem is similar to that discussed on page 300 dealing with the ratio of potency between two preparations at different levels of sensitivity.

The significance of these results for standardization should be clear. Within the range of 1 to 10 TU, a dose-response curve can be approximated after determination of two points, and the dose which will produce a given mean response can then be estimated by interpolation or extrapolation. The more "positive" reactors there are in the test group, the steeper the curve and the more accurate the inference from a given response to the corresponding dose.

### *Comparison of PPD-S and RT XIX-XX-XXI*

The method suggested here for standardization of tuberculin in human subjects follows the same general design described for the preliminary dose-response studies. Various doses of an unknown tuberculin are compared with a suitable fixed control dose of the tuberculin selected as the standard. The dose of the unknown tuberculin which gives the same mean response as the standard control dose is then estimated by interpolation or extrapolation.

For the purpose of this study, *RT XIX-XX-XXI* (hereafter denoted as *RT*) was designated as the "standard" because the dose-response relationship had already been determined. Ten tuberculin units were considered as an appropriate fixed control dose of this preparation. While PPD-S is, in fact, accepted as the international standard PPD tuberculin, it is considered here as the "unknown" preparation for the purpose of illustrating the method. Preliminary studies carried out at Statens Seruminstitut on guinea pigs and in a small number of humans had indicated that *PPD-S* probably was stronger than *RT* weight for weight. Accordingly, the following three specific doses were selected for PPD-S: 0.00004 mg; 0.00012 mg., and 0.0002 mg. of the purified substance, corresponding to 2, 6, and 10 TU. It was expected that the equivalent to the 10 TU control dose of RT would fall within this range.

This first part of the comparison of PPD-S and RT was carried out in June, 1951, on school children in a rural district of Denmark. The study group comprised 544 children, 7 to 15 years of age (277 males and 267 females), all previously BCG vaccinated, mostly during 1948 to 1950.

The results are presented in detail in tables 2, 3, and 4, showing the correlation between the sizes of induration to 10 TU (0.0002 mg.) of RT and the specified dose of PPD-S for each sub-group.

The mean and the variance[6] of the sizes of induration have been computed for both doses in each sub-group (table 5). Figure 3 graphically represents the deviation of the mean induration to each specified dose of PPD-S from the mean induration to 10 TU of RT.

The point where this dose-response curve intersects the abscissa represents an estimate of the dose of PPD-S which gives the same mean response as 10 TU of RT. A straight line fitted to all three points of the curve, indicated by a dotted line in the figure, intersects the abscissa at the point 9.4 TU. The standard error of this estimation is approximately 0.3 TU. If the interpolation is based only on the points from the two sub-groups tested with 6 and 10 TU of PPD-S, the in-

[6] The variance is the square of the standard deviation and thus a measure of the dispersion of the distribution.

tersection is at the point 9.8 TU, also with a standard error of approximately 0.3 TU. Provided the two preparations are qualitatively equal, their ratio of potency should be very close to unity.

The next question is whether the same ratio will be obtained in test subjects with different degrees of sensitivity. Actually, a separation of individual persons into different degrees of sensitivity would require an additional tuberculin test, independent of those used for comparing the two preparations. The problem,

TABLE 2

183 BCG-Vaccinated School Children Distributed by Size of Induration to Mantoux Tests with 2 TU of PPD-S and 10 TU of RT XIX-XX-XXI

| Size of Induration in Mm. to 2 TU of PPD-S | Size of induration in mm. to 10 TU of RT XIX-XX-XXI | | | | | | | | | | | | | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | |
| 0 | | 1 | | | | 1 | | | | | | | | | | | | | | | | | | | | 2 |
| 1 | | | | | | | 1 | 1 | | | | | | | | | | | | | | | | | | 2 |
| 2 | | | | | | 1 | | | 1 | 1 | | | | | | | | | | | | | | | | 3 |
| 3 | | | | 1 | 1 | 1 | 1 | | | 2 | | | 1 | 2 | | 1 | | | | | | | | | | 10 |
| 4 | | | | 1 | | 3 | 2 | 1 | | 1 | 2 | 2 | | | | 2 | | 1 | | | | | | | | 15 |
| 5 | | | | | 1 | | 4 | 1 | | 2 | | 3 | 1 | | 1 | 2 | | 1 | | | | | | | | 16 |
| 6 | | | | | | | | | 1 | 2 | 2 | | 1 | 4 | 1 | 3 | 3 | 1 | | | | | | | | 18 |
| 7 | | | | | | | | | 1 | | 1 | 1 | | 3 | 2 | 1 | 4 | | | 1 | | | | | | 14 |
| 8 | | | | | | | | | | 2 | | | 1 | | 1 | 2 | 3 | 1 | 2 | | 1 | | | | | 13 |
| 9 | | | | | | | | | | | | | 1 | 1 | | 2 | 3 | 1 | 2 | | 2 | | 1 | | | 13 |
| 10 | | | | | | | | | | | | | | 1 | | | 1 | | 2 | 2 | | 1 | | | | 7 |
| 11 | | | | | | 1 | | | | | 1 | | | | | | 1 | | 1 | 3 | 2 | | | | | 9 |
| 12 | | | | | | | | | | | | | | | | 1 | | 1 | 1 | 1 | 1 | 1 | | | | 6 |
| 13 | | | | | | | | | | | | | | 1 | | 1 | 1 | | 3 | 2 | 1 | 1 | | | | 10 |
| 14 | | | | | | | | | | | | | 1 | | 1 | | 1 | 1 | 5 | 1 | | 1 | 1 | | | 12 |
| 15 | | | | | | | | | | | | | | | | | 1 | | 1 | 3 | 1 | 1 | | 1 | 1 | 9 |
| 16 | | | | | | | | | | | | | | | | | | 1 | 1 | 1 | 3 | 1 | | | | 7 |
| 17 | | | | | | | | | | | | | | | | | 1 | | | 1 | 1 | 1 | 2 | | | 6 |
| 18 | | | | | | | | | | | | | | | | | | 1 | | 1 | 1 | | 2 | 1 | | 6 |
| 19 | | | | | | | | | | | | | | | | | | | | | 2 | | | | | 2 |
| 20 | | | | | | | | | | | | | | | | | | | | | 2 | 1 | | | | 3 |
| Total.... | | 1 | | 2 | 2 | 7 | 8 | 3 | 3 | 10 | 6 | 6 | 6 | 12 | 6 | 15 | 19 | 9 | 18 | 16 | 17 | 8 | 6 | 2 | 1 | 183 |

however, can be studied indirectly from the correlation of the results, obtained by two (presumably) equivalent doses.

If the distribution is everywhere symmetrical about the diagonal of equal reactions, with the same range of reactions for both doses, the two doses correspond to each other on all levels of sensitivity. Figure 4*A* illustrates such a distribution schematically. Suppose, however, that one of the preparations is less potent than the other in highly sensitive individuals but correspondingly more potent for individuals with low sensitivity (so that the average response in the entire group is the same). The reactions obtained from this preparation would have a smaller range; the two extremes in the group would be pulled together.

The distribution would be asymmetrical with respect to the diagonal, as in figure 4*B*.

An asymmetrical correlation can be demonstrated numerically in various ways. Except for very irregular distributions, asymmetry will generally produce different dispersions of the marginal distributions. A comparison of the two variances will, therefore, provide a simple expression of the degree of asymmetry.

TABLE 3

181 BCG-Vaccinated School Children Distributed by Size of Induration to Mantoux Tests with 6 TU of PPD-S and 10 TU of RT XIX-XX-XXI

| Size of Induration in Mm. to 6 TU of PPD-S | Size of induration in mm. to 10 TU of RT XIX-XX-XXI | | | | | | | | | | | | | | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | |
| 0 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | | | | | | | | | 1 | | | | | | | | | | | | | | | | | | 1 |
| 4 | | | | 2 | | | | | 1 | | 1 | | | | | | | | | | | | | | | | 4 |
| 5 | | | | | | 1 | 2 | 1 | 1 | 2 | | | 1 | 1 | | | | | | | | | | | | | 9 |
| 6 | | | | | | 1 | | 1 | | | | | | 1 | | | | | | | | | | | | | 3 |
| 7 | | | | | | 2 | 1 | | | 2 | | | 1 | 2 | | | | | | | | | | | | | 8 |
| 8 | | | | | | | | 1 | | 1 | 2 | 4 | | 1 | | | | | | | | | | | | | 9 |
| 9 | | | | | | | 1 | | | | 2 | 1 | | 1 | 1 | | | 1 | | | | | | | | | 7 |
| 10 | | | | | | | | | | | 2 | | 1 | 3 | 2 | | 2 | 1 | 1 | | | | | | | | 12 |
| 11 | | | | | | | | | | 1 | | 1 | 1 | 1 | | | 1 | | 1 | | | | | | | | 6 |
| 12 | | | | | | | | | | | | | 1 | 1 | 3 | 1 | 2 | 2 | | | 1 | 1 | | | | | 12 |
| 13 | | | | | | | | | | | 1 | 1 | 3 | | | 1 | 3 | | 3 | 2 | 1 | | | | | | 15 |
| 14 | | | | | | | | | | | 1 | | | 1 | 2 | 2 | 3 | | 2 | | 1 | 1 | | | | | 13 |
| 15 | | | | | | | | | | | | | | | 2 | | 4 | 3 | 5 | | 2 | | | | | | 16 |
| 16 | | | | | | | | | | | | | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | | | 1 | | 13 |
| 17 | | | | | | | | | | | | | | | 2 | 1 | 2 | 1 | | 4 | 2 | 1 | | | | | 13 |
| 18 | | | | | | | | | | | | | | | | | 1 | 1 | | 3 | 2 | 2 | 1 | 1 | | | 11 |
| 19 | | | | | | | | | | | | | | | | 1 | | 2 | 3 | 2 | | 3 | | | | | 11 |
| 20 | | | | | | | | | | | | | | | | | 1 | 1 | 3 | 1 | 2 | 2 | | | | | 10 |
| 21 | | | | | | | | | | | | | | | | | 1 | 1 | | | 1 | 1 | | 1 | | | 5 |
| 22 | | | | | | | | | | | | | | | | | | | | | 1 | | | | | | 1 |
| 23 | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | 1 |
| 24 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 25 | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | 1 |
| Total.... | | | | 2 | | 4 | 4 | 3 | 3 | 6 | 9 | 7 | 9 | 13 | 13 | 7 | 21 | 15 | 20 | 13 | 14 | 13 | 1 | 2 | 1 | 1 | 181 |

The variances of the marginal distributions for all doses used in this study are given in table 5. That the reactions produced by 10 TU of PPD-S and 10 TU of RT in the third sub-group have exactly the same variance confirms the impression of symmetry about the diagnonal of equal reactions seen on inspection of table 4.

The preceding problem concerning the ratio of potency on different levels of sensitivity must be clearly distinguished from a variation of the ratio of potency

TABLE 4

180 BCG-Vaccinated School Children Distributed by Size of Induration to Mantoux Tests with 10 TU of PPD-S and 10 TU of RT XIX-XX-XXI

| Size of Induration in Mm. to 10 TU of PPD-S | Size of Induration in mm. to 10 TU of RT XIX-XX-XXI | | | | | | | | | | | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | |
| 0 | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | | | | | 1 | | | | | | | | | | | | | | | | | | | 1 |
| 4 | | 1 | | 1 | | | 1 | 1 | | | | | | | | | | | | | | | | 4 |
| 5 | | | | 1 | 1 | 1 | | | 1 | | | | | | | | | | | | | | | 4 |
| 6 | | | | | | 2 | 1 | 2 | | | | | | | | | 1 | | | | | | | 6 |
| 7 | | | | 1 | | | | 3 | 1 | 1 | | | | | 1 | | | | | | | | | 7 |
| 8 | | | | | | | | 1 | | | | | 1 | | | | | | | | | | | 2 |
| 9 | | | | | | | | 1 | 1 | | | | | | | | | | | | | | | 2 |
| 10 | | | | | | | | 1 | | 2 | | | 1 | | 1 | | | | | | | | | 5 |
| 11 | | | | | | | | 1 | 1 | 1 | 4 | 1 | 2 | | | 1 | 1 | | | | | | | 12 |
| 12 | | | | | | | | | | | | 1 | 2 | 2 | | | 2 | | | | | | | 7 |
| 13 | | | | | | | | | | 1 | | 2 | 3 | | | 2 | | | | 1 | 1 | | | 10 |
| 14 | | | | | | | | | | | 1 | | | 1 | 1 | 2 | 2 | | 1 | | | | | 8 |
| 15 | | | | | | | | | | | | 1 | | 1 | 2 | 4 | 3 | 5 | 1 | 3 | | | | 20 |
| 16 | | | | | | | | | | 1 | | | | 5 | 1 | 4 | 5 | 1 | 2 | | | | | 19 |
| 17 | | | | | | | | | | | | | | 1 | 1 | | | 1 | 3 | 1 | 1 | | | 8 |
| 18 | | | | | | | | | | | | | | 2 | | | 4 | 6 | 3 | 1 | 1 | 2 | | 19 |
| 19 | | | | | | | | | | | | | | | | 2 | | 1 | 2 | 2 | 6 | 2 | 1 | 16 |
| 20 | | | | | | | | | | | | | | | | | | 4 | 2 | 1 | 3 | 4 | | 14 |
| 21 | | | | | | | | | | | | | | | | | | | 3 | 3 | 5 | | | 11 |
| 22 | | | | | | | | | | | | | | | | | | | | | 2 | | 1 | 3 |
| 23 | | | | | | | | | | | | | | | | | | | | | 2 | | | 2 |
| Total.... | | 1 | | 3 | 2 | 3 | 2 | 10 | 4 | 6 | 5 | 5 | 9 | 12 | 7 | 15 | 18 | 18 | 17 | 12 | 21 | 8 | 2 | 180 |

TABLE 5

Means and Variances of Sizes of Indurations to Mantoux Tests with Various Doses of PPD-S and with 10 TU of RT XIX-XX-XXI in BCG-Vaccinated School Children

| Sub-group tested with specific dose | Number of persons tested | Mean size of induration to: | | Variances of sizes of induration to: | |
|---|---|---|---|---|---|
| | | Specific Dose of PPD-S | 10 TU of RT XIX-XX-XXI | Specific Dose of PPD-S | 10 TU OF RT XIX-XX-XXI |
| 2 TU of PPD-S | 183 | 9.28 | 14.70 | 23.63 | 26.37 |
| 6 TU of PPD-S | 181 | 13.43 | 15.18 | 22.72 | 20.49 |
| 10 TU of PPD-S | 180 | 14.86 | 14.74 | 22.66 | 22.67 |

with the size of doses used for comparison. Even after having demonstrated, for all levels of sensitivity, a complete equivalence between two doses, e.g., 10 TU of PPD-S and RT, it may be questioned whether this will hold for fractions or

multiples of both doses. A comparison of the slopes of the dose-response curves for the two preparations will give some information about this question. If the slopes are different (in comparable groups), the same ratio of potency cannot be used in different parts of the dosage scale. A thorough study of the problem requires, however, that, after the first determination of two equivalent doses, additional pairs of presumably equivalent doses be selected from various parts of the dosage scale. The results must be correlated for each pair, and the ques-

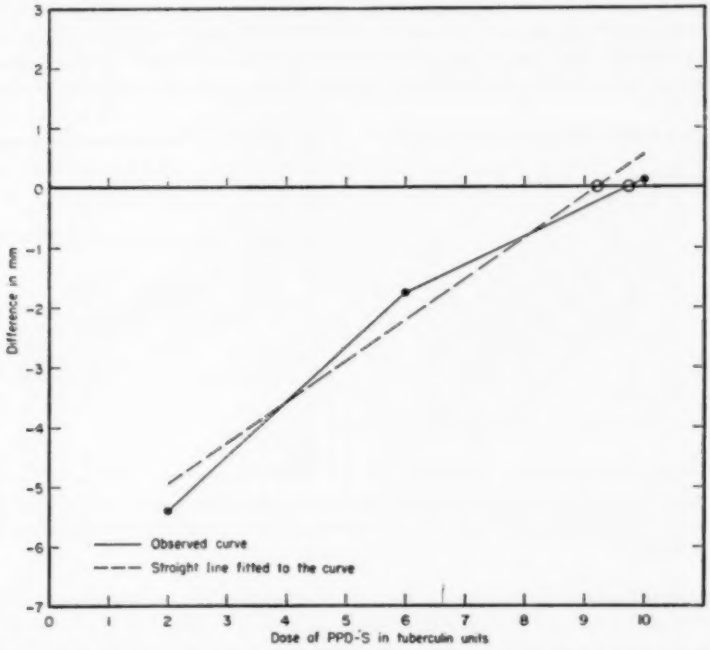


FIG. 3. Differences between mean sizes of induration to Mantoux tests with a specified dose of PPD-S and with 10 TU of RT XIX-XX-XXI (1 TU = 1/50,000 mg. for both products) among 544 BCG-vaccinated school children.

tion of equivalence in mean response and for separate levels of sensitivity must then be considered as before.

Because of limited facilities for additional testing with higher doses, the investigation was confined to a comparison of 100 TU (0.002 mg.) of both preparations, which could be expected to be approximately equivalent. The test group for this comparison consisted of all the non-reactors from several concurrent studies in which 10 TU of RT were being used. From a total of 990 children in these several studies, 195 (109 males and 86 females) who reacted with less

than 6 mm. of induration to the 10 TU test comprised the group tested with the higher doses. All were between 7 and 15 years of age; 108 had been previously BCG-vaccinated, 89 in 1948.

The result of this comparison is given in tables 6 and 7. It is seen that the means are practically equal, the difference being only 0.15 mm. with a standard error of 0.22 mm. In other words, a ratio of potency of about unity also fits the *mean response* to the 100 TU dose of these tuberculins.

The variance, however, is somewhat greater for the reactions produced by 100 TU of RT than for those produced by 100 TU of PPD-S, and the difference is statistically significant. A large dose of RT thus seems to give a greater dispersion of the reactions than the corresponding dose of PPD-S. This can also be seen by direct inspection of the correlation table (table 6). Among individuals

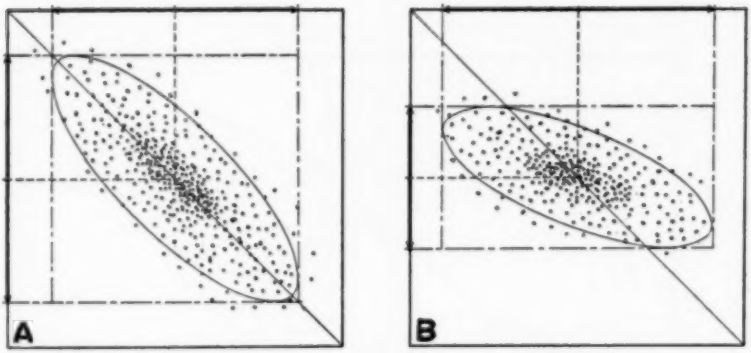


FIG. 4. Schematic illustration of correlation diagrams resulting from: *A.* two equivalent doses; *B.* two doses giving equal mean responses, without being equivalent for every level of sensitivity.

with small reactions, localized in the upper left-hand corner of the table, a majority react somewhat stronger to PPD-S than to RT (most of them are below the diagonal of equal reactions). In contrast to this, most persons with larger reactions react somewhat weaker to PPD-S (they are mostly situated above the diagonal). The distribution corresponds to that indicated in figure 4*B* and implies that reactions become more concentrated by the use of 100 TU of PPD-S, more dispersed by the use of 100 TU of RT.

A further clarification of this finding was obtained by separation of the 108 previously BCG-vaccinated from the 80 non-vaccinated children (in 7 cases information was lacking). The results are given in table 8 and tables 9 and 10.

It appears that 100 TU of RT give larger reactions than 100 TU of PPD-S in BCG-vaccinated children with a mean induration of 13.42 mm. against 12.59 mm. In contrast, PPD-S was stronger in non-vaccinated children, giving a mean induration of 5.58 mm. against 4.94 mm. for RT. The differences between the

means are statistically significant in both cases. The implications of this finding will be discussed in the next section.

TABLE 6

195 School Children Distributed by Size of Induration to Mantoux Tests with 100 TU of PPD-S and 100 TU of RT XIX-XX-XXI

| Size of Induration in Mm. to 100 TU of PPD-S | Size of Induration in mm. to 100 TU of RT XIX-XX-XXI | | | | | | | | | | | | | | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | |
| 0 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | | | 2 | | | | | | | | | | | | | | | | | | | | | | | | | 2 |
| 2 | | 1 | | 1 | | | | | | | | | | | | | | | | | | | | | | | | 2 |
| 3 | | 1 | 3 | 6 | 5 | 2 | 2 | 1 | 1 | | | | | | | | | | | | | | | | | | | 21 |
| 4 | | 1 | 1 | 5 | 8 | 3 | 1 | | | | | | | | | | | | | | | | | | | | | 19 |
| 5 | | 1 | | 5 | 1 | 2 | 1 | | | | | | | | | | | | | | | | | | | | | 10 |
| 6 | | | 2 | 2 | | 4 | 2 | 3 | | 1 | | 1 | | | | | | | | | | | | | | | | 15 |
| 7 | | | | | 4 | 2 | 2 | 1 | 2 | 1 | | | | | | | | | | | | | | | | | | 12 |
| 8 | | | | | 1 | 1 | 3 | 1 | 3 | 3 | 2 | 1 | | 1 | | | | | | | | | | | | | | 16 |
| 9 | | | | 1 | 1 | | | 1 | | 2 | 1 | 1 | 2 | 1 | | | | | 1 | | | | | | | | | 11 |
| 10 | | | | | | | | | | 1 | 2 | 2 | 1 | 3 | 2 | 1 | | | | | | | | | | | | 12 |
| 11 | | | | | | | | | | | 1 | 1 | 2 | 2 | | 1 | | | | | | | | | | | | 7 |
| 12 | | | | | | | | | | | | 1 | 1 | 2 | | 2 | 1 | | 1 | 1 | | | | | | | | 9 |
| 13 | | | | | | | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | | | | | | | | | 8 |
| 14 | | | | | | | | | | | | | 2 | 1 | | 2 | 2 | 1 | 1 | | | | | | | | | 9 |
| 15 | | | | | | | 1 | | | | | 1 | | 1 | 3 | 2 | | 1 | | | | | | | | | | 9 |
| 16 | | | | | | | | | | | | | | | | 3 | 4 | 3 | 1 | | | | | | | | | 11 |
| 17 | | | | | | | | | | | | | | | | 2 | 1 | | | | 1 | | | | 1 | | | 5 |
| 18 | | | | | | | | | | | | | 1 | | | 1 | 3 | 2 | 2 | 2 | | | | | | | | 11 |
| 19 | | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | 2 |
| 20 | | | | | | | | | | | | | | | | | 1 | | 1 | | | | | | | | | 2 |
| 21 | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | 1 |
| . | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| . | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| . | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 27 | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | 1 |
| Total.... | | 4 | 8 | 20 | 20 | 14 | 12 | 7 | 6 | 8 | 7 | 9 | 10 | 12 | 6 | 15 | 13 | 9 | 8 | 3 | 1 | | 1 | | 1 | | 1 | 195 |

TABLE 7

Means and Variances of Sizes of Indurations to Mantoux Tests with 100 TU of PPD-S and 100 TU of RT XIX-XX-XXI in 195 School Children

| Preparation | Mean Size of Induration | Variance of Sizes of Induration |
|---|---|---|
| PPD-S | 9.49 | 26.94 |
| RT XIX-XX-XXI | 9.64 | 31.62 |

A question not yet considered is the degree to which reactions produced by equivalent doses can differ in individual cases. Great disagreement could occur between the two tests performed on each single person without influencing the

TABLE 8

MEANS AND VARIANCES OF SIZES OF INDURATIONS TO MANTOUX TESTS WITH 100 TU OF PPD-S AND 100 TU OF RT XIX-XX-XXI IN 108 BCG-VACCINATED AND 80 NON-VACCINATED SCHOOL CHILDREN

| PREPARATION | MEAN SIZE OF INDURATION IN: | | VARIANCE OF SIZES OF INDURATION IN: | |
|---|---|---|---|---|
| | BCG-Vaccinated Children | Non-Vaccinated Children | BCG-Vaccinated Children | Non-Vaccinated Children |
| PPD-S | 12.59 | 5.58 | 18.97 | 9.89 |
| RT XIX-XX-XXI | 13.42 | 4.94 | 18.02 | 8.49 |
| Difference | −0.83 | +0.64 | | |

TABLE 9

108 BCG-VACCINATED SCHOOL CHILDREN DISTRIBUTED BY SIZE OF INDURATION TO MANTOUX TESTS WITH 100 TU OF PPD-S AND 100 TU OF RT XIX-XX-XXI

| SIZE OF INDURATION IN MM. TO 100 TU OF PPD-S | SIZE OF INDURATION IN MM. TO 100 TU OF RT XIX-XX-XXI | | | | | | | | | | | | | | | | | | | | | | | | | TOTAL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | |
| 0 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | | | 1 | | | 1 | 1 | | 1 | | | | | | | | | | | | | | | | | | | 4 |
| 4 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| 6 | | | | | | | 1 | 1 | | | | 1 | | | | | | | | | | | | | | | | 3 |
| 7 | | | | | | | | | 1 | 1 | | | | | | | | | | | | | | | | | | 2 |
| 8 | | | | | | | 1 | | 3 | 2 | 2 | 1 | | 1 | | | | | | | | | | | | | | 10 |
| 9 | | | | 1 | | | | | | 2 | | 1 | | 1 | | | | | 1 | | | | | | | | | 6 |
| 10 | | | | | | | | | | 1 | 2 | 2 | 1 | 3 | 2 | 1 | | | | | | | | | | | | 12 |
| 11 | | | | | | | | | | | 1 | 1 | 2 | 2 | | 1 | | | | | | | | | | | | 7 |
| 12 | | | | | | | | | | | | 1 | 1 | 2 | | 2 | 1 | | 1 | 1 | | | | | | | | 9 |
| 13 | | | | | | | | | | | | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | | | | | | | | | 7 |
| 14 | | | | | | | | | | | | | 1 | 1 | | 2 | 2 | 1 | 1 | | | | | | | | | 8 |
| 15 | | | | | | | | | | | | 1 | | 1 | 3 | 2 | | 1 | | | | | | | | | | 8 |
| 16 | | | | | | | | | | | | | | | | 3 | 4 | 3 | 1 | | | | | | | | | 11 |
| 17 | | | | | | | | | | | | | | | | 2 | 1 | | | | 1 | | | | 1 | | | 5 |
| 18 | | | | | | | | | | | | | 1 | | | | 3 | 2 | 1 | 2 | | | | | | | | 9 |
| 19 | | | | | | | | | | | | | | | | | | 2 | | | | | | | | | | 2 |
| 20 | | | | | | | | | | | | | | | | | 1 | | 1 | | | | | | | | | 2 |
| 21 | | | | | | | | | | | | | | | | | | | | | | | 1 | | | | | 1 |
| . | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| . | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| . | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 27 | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 | 1 |
| Total | | 1 | 1 | 1 | | 1 | 3 | 1 | 5 | 6 | 5 | 9 | 7 | 12 | 6 | 14 | 13 | 9 | 7 | 3 | 1 | | 1 | | 1 | | 1 | 108 |

means and variances. It would, however, be reflected by an excessive dispersion from the diagonal in the correlation diagram. Such poor equivalence of the products in individual cases would mean that the reactions in each case were determined largely by experimental errors and only to a small extent by a property (the sensitivity) of the subject. It is reasonable to require that individual disagreements resulting from equivalent doses of two preparations should not exceed those obtained from equal doses of the same preparation.

TABLE 10

80 NON-VACCINATED SCHOOL CHILDREN DISTRIBUTED BY SIZE OF INDURATION TO MANTOUX TESTS WITH 100 TU OF PPD-S AND 100 TU OF RT XIX-XX-XXI

| SIZE OF INDURATION IN MM. TO 100 TU OF PPD-S | SIZE OF INDURATION IN MM. TO 100 TU OF RT XIX-XX-XXI | | | | | | | | | | | | | | | | | | TOTAL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | |
| 0 | | | | | | | | | | | | | | | | | | | | |
| 1 | | | 2 | | | | | | | | | | | | | | | | | 2 |
| 2 | | 1 | | 1 | | | | | | | | | | | | | | | | 2 |
| 3 | | 1 | 2 | 6 | 4 | 1 | 1 | 1 | | | | | | | | | | | | 16 |
| 4 | | 1 | | 4 | 8 | 3 | 1 | | | | | | | | | | | | | 17 |
| 5 | | | | 5 | 1 | 2 | 1 | | | | | | | | | | | | | 9 |
| 6 | | | 1 | 2 | | 4 | 1 | 1 | | 1 | | | | | | | | | | 10 |
| 7 | | | | | 4 | 2 | 2 | 1 | 1 | | | | | | | | | | | 10 |
| 8 | | | | | 1 | | 2 | 1 | | 1 | | | | | | | | | | 5 |
| 9 | | | | | 1 | | | 1 | | | 1 | | 2 | | | | | | | 5 |
| 10 | | | | | | | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | 1 | | | | | | | | | 1 |
| 14 | | | | | | | | | | | | | | | | | | | | |
| 15 | | | | | | | 1 | | | | | | | | | | | | | 1 |
| 16 | | | | | | | | | | | | | | | | | | | | |
| 17 | | | | | | | | | | | | | | | | | | | | |
| 18 | | | | | | | | | | | | | | | | 1 | | | 1 | 2 |
| Total.... | | 3 | 5 | 18 | 19 | 12 | 9 | 5 | 1 | 2 | 2 | | 2 | | | 1 | | | 1 | 80 |

A measure of the disagreement in individual cases can be given in different ways. One method is to distribute the subjects according to the difference between the two tests and compute the variance for this distribution. A variance of 5.95 is obtained for the differences between reactions produced by 10 TU of PPD-S and 10 TU of RT. As shown in a previous report (6), variances between 5.47 and 10.28 have been found in corresponding distributions by use of equal doses of the same product (10 TU or 5 TU of RT). The agreement in individual cases between reactions produced by 10 TU of PPD-S and 10 TU of RT is, therefore, not less than between reactions produced by equal doses of RT.

## DISCUSSION

The present report has two purposes. First, it describes a design for standardizing tuberculin preparations, specifically with respect to their properties for

producing skin reactions in humans. Second, it presents the results of a comparison of two important purified tuberculins: PPD-S produced in the United States under the direction of Dr. Florence Seibert, and RT XIX-XX-XXI produced by Statens Seruminstitut in Copenhagen.

The method proposed here is based on duplicate intradermal Mantoux tests, given symmetrically on the two arms of human subjects, with randomization of doses and preparations according to a certain scheme. The test subjects consisted of school children, most of whom had been previously BCG vaccinated.

The use of duplicate tests in contrast to single tests has the advantage of requiring fewer test subjects. Each pair of reactions reflects a common level of sensitivity; thus, if their differences are used to study the effect of a certain factor, then the standard error of the result will depend only on experimental errors in giving and reading tests. On the other hand, a comparison of two preparations on different individuals, each of whom received a single test, entails a greater standard error because of random variations in the level of sensitivity. Moreover, the use of single tests would not permit a direct correlation of the result produced by two preparations.

The use of more than two tests on each individual is hardly practicable on a great number of persons and might also involve a danger of interaction between tests placed too close together.

The test subjects should include a high proportion of "positive" reactors in order to benefit from a steep dose-response curve. In the present project, BCG-vaccinated school children were chosen partly for this reason and partly because the studies could be done in connection with routine re-examinations for post-vaccination allergy. It is advisable, however, to compare the preparations on vaccinated persons as well as on naturally infected persons, as the comparisons may give different results in the two groups. A difference similar to that discovered between PPD-S and RT at the 100 TU level might have been found also at the 10 TU level, if non-vaccinated children had been included in the comparison.

With respect to the doses selected for comparison, certain points should be considered: First, the comparison should include doses which are commonly used in practical work, i.e., approximately 5 to 10 TU. Next, the dose-response curve shows that, within the range of 1 to 10 TU, the higher doses provide a more accurate assay because a given change in concentration of the product produces a greater change in response. For these reasons 10 TU were chosen as an appropriate fixed dose of the standard tuberculin in the present investigation.

In order to find the equivalent dose of the other preparation, various selected doses are tested against this standard dose. In the present study, three different doses of the unknown preparation were selected for comparison, but two may often be preferred in order to simplify the design and increase the number of observations in each comparison. How shall these doses be selected?

An essential assumption for the method is that the dose-response curve for the unknown tuberculin is close to a straight line. The substitution of this curve by a straight line will, naturally, be more uncertain over a wide dosage

range than over a short dosage range, since the central part of the curve may deviate considerably from the straight line connecting the two points. For this reason it is desirable to make the interval between the doses small. On the other hand, it should not be so small that the equivalent dose falls outside, rather than within, the dose span, because this gives a much greater standard error of the estimated equivalent. The choice of doses, therefore, must be based on the extent to which this equivalent can be anticipated by previous knowledge of the preparations. In the absence of such knowledge, it might be profitable to carry out a preliminary assay with a wide range of doses, followed by an accurate standardization with adjusted doses.

There is another limitation, however, as to how small the interval between the selected doses can be. The closer the points are together, the more uncertain is the use of the observed slope for estimating the unknown equivalent dose. Suppose that the unknown equivalent lies between the two selected doses, but that the dose span is so small that random errors can cause both observed responses to fall either above or below the abscissa. It is clear that the intersection of the abscissa and the straight line derived from these responses could occur very far from the correct value. In such cases (namely, with too small differences between the selected doses) it would be better to disregard the observed slope for the unknown preparation and to replace it by the one predetermined for the standard tuberculin. This would entail considerable uncertainty, however, and important information about the slope of the new tuberculin would be lacking.

How great the difference between the two doses must be in order to justify use of the observed slope for the unknown preparation depends on the standard error of the individual response and on the number of test subjects. The standard error of the estimated slope is $\frac{s}{d}\sqrt{\frac{2}{N}}$, $s$ being the standard error of the individual response, $d$ the difference between the two selected doses, and $N$ the number of subjects used for testing each dose. Each individual response is a difference between two tests on the same person. In this study the standard error of this difference was 2.4 mm., 3 mm., and 3.5 mm. in the three sub-groups tested with 10 TU, 6 TU, and 2 TU of PPD-S. Assuming the value 3 mm. for $s$, it is found that the use of 50, 100, or 200 subjects for each dose requires differences of 4 TU, 3 TU, or 2 TU between the doses in order to give a standard error of 0.15 mm. per TU in the estimated slope. This can be regarded as satisfactory accuracy when the true slope is approximately 1 mm. per TU, as was actually found for RT in the neighborhood of 5 TU. If the true slope were smaller, greater accuracy would be needed for its estimation.

The *standard error of the estimated equivalent dose* depends on the number of test subjects, on the steepness of the dose-response curve, and on the location of the true equivalent in relation to the doses selected for comparison. Assuming that the two doses differ sufficiently to allow use of the observed slope for the unknown tuberculin, the result can be summarized briefly as follows: The standard error is smallest if the true equivalent is in the center of the range of doses used. It is then equal to $\frac{s}{b\sqrt{N}}$ TU, where $s$ and $N$ have the same meaning as before, while $b$ is the slope in mm. per TU for the unknown preparation. The standard error increases with increasing deviation of the equivalent from

the center of the dose span and reaches the value $\frac{s}{b}\sqrt{\frac{2}{N}}$ TU when the equivalent coincides with one of the selected doses. A shift of the equivalent dose outside the dose span causes a further and rapid increase in the standard error.

Assuming a slope of $b = 1$ mm. per TU for the unknown preparation, 50 test subjects for each dose give a standard error of 0.42 TU, provided the equivalent falls in the middle of the dose span, but 0.60 TU if it coincides with one of the selected doses. One hundred persons give corresponding standard errors of 0.30 TU and 0.43 TU and 200 persons give 0.21 TU and 0.30 TU. If the test doses are approximately 10 TU, a change of 1 TU will mean 10 per cent change in strength of dilution, so that the standard errors given above in tuberculin units correspond to errors of 2.1 to 6.0 per cent in strength of dilution. Thus, 100 subjects may be considered as an appropriate number for each dose, requiring at least 3 TU difference between the doses and estimating the unknown equivalent with a standard error of 3.0 to 4.3 per cent (differing according to its location in the dose span).

Once the dose of the unknown tuberculin which gives the same mean response as the fixed standard dose has been determined with sufficient accuracy, problems of qualitative differences need further consideration before the doses can be declared equivalent. The first question is whether the doses give identical results on each level of sensitivity. A second question is whether multiples or fractions of the two doses are equivalent. Finally, it may be asked how well two presumably equivalent doses agree in individual cases, i.e., in giving consistent results in each particular person. A study of these problems requires additional series of duplicate tests, each series performed with two doses expected to be equivalent according to the first comparison. Within each series, the two means, the variances, and the correlation diagram will provide information concerning the equivalence of the two doses used.

The comparison of PPD-S and RT showed that 10 TU or 0.0002 mg. of the two substances gave approximately the same mean response and also could be regarded as identical for separate levels of sensitivity in groups of BCG-vaccinated school children. The slope of the dose-response curve for the two tuberculins was also approximately the same, each unit giving approximately 0.8 mm. increase in size of induration.

Small, but statistically significant, differences were, however, revealed by direct comparison of 100 TU or 0.002 mg. of the two preparations in a mixed group of BCG-vaccinated and non-vaccinated children, all of whom were non-reactors to 10 TU of RT. PPD-S gave slightly stronger reactions in the children with a very low degree of sensitivity, the non-vaccinated group. Conversely, 100 TU of the RT proved to be a little more potent in those with a slightly higher level of sensitivity, the BCG-vaccinated children.

The statistically significant but rather small difference between these two preparations is of little practical importance in their everyday use. The finding is pertinent in other respects, however, as it shows that the two purified substances are not identical, nor can they be regarded simply as having slightly different concentrations of an identical principle. PPD-S must contain a higher

proportion of some factor which produces a response in children who have a very low degree of sensitivity. RT, on the other hand, must contain more of the factor responsible for reactions in children with a higher level of sensitivity.

Moreover, this finding is useful as an illustrative analogy to results from other investigations concerning the sources of tuberculin sensitivity in various populations. In the present report, the existence of two groups, suggested by the responses to the two tuberculins, was found to correspond to a division of the children according to the sensitizing antigen (BCG). Even in the absence of knowledge of the source of sensitivity, however, an asymmetrical correlation of responses to two preparations may suggest that the population under test has been sensitized by different antigens. Qualitatively different preparations may thus provide the tools with which to study the nature and specificity of tuberculin sensitivity.

## SUMMARY

A method is described for standardization of tuberculin preparations with respect to their properties of producing skin reactions in humans. The principal steps in the method are:

1. An appropriate dose of the standard preparation is chosen, preferably within the range commonly used for practical work.
2. Various doses of the unknown preparation are compared with the standard by duplicate Mantoux tests.
3. The dose of the unknown giving the same mean response as the standard is determined and is tentatively called the equivalent dose.
4. Pairs of doses expected to be equivalent are selected from various parts of the dosage scale and compared directly by duplicate tests. The correlated results for each pair provide data for study of qualitative differences between the products.
5. Doses from two preparations can be considered equivalent only if they produce (*a*) the same mean response, (*b*) a symmetrical distribution around the diagonal of equal reactions, and (*c*) a dispersion from this diagonal not greater than that obtained from two equal doses of one preparation.
6. If multiples of a pair of equivalent doses always prove to be equivalent, then the two preparations may be considered qualitatively equal. The constant ratio between equivalent doses is then called the ratio of potency between the preparations.

A comparison was made of two purified tuberculins, PPD-S from the United States and RT XIX-XX-XXI from Denmark. Doses of 0.0002 mg. of each were found to be equivalent when given to BCG-vaccinated school children.

Ten times this dose (0.002 mg.) from both preparations produced equal mean responses in a mixed group of vaccinated and non-vaccinated children who were non-reactors to the lower dose of RT. PPD-S was more potent than RT in the non-vaccinated children, however; whereas the reverse result was found in the vaccinated group. A slight qualitative difference was therefore demonstrated

between the preparations, corresponding to the source of sensitivity (BCG) among the children.

In human populations sensitized from unknown sources, qualitatively different preparations may be used to disclose the existence of different sensitizing agents.


## SUMARIO

*Método para la Uniformización de las Preparaciones de Tuberculina Mediante Intracutirreacciones en Seres Humanos. Comparación de Dos Tuberculinas Purificadas*

El método descrito permite uniformizar las preparaciones de tuberculina a base de sus propiedades de producir cutirreacciones en el hombre. Los principales tiempos de la técnica son:

1. Se escoge una dosis apropiada de la preparación tipo, preferiblemente dentro de los límites utilizados habitualmente en la práctica.
2. Compáranse varias dosis de la preparación desconocida con el tipo, empleando para ello reacciones de Mantoux en duplicado.
3. Determínase la dosis de la preparación comprobada que evoque la misma respuesta media que la tipo, designándosela tentativamente como dosis equivalente.
4. Tómanse de varias partes de la escala de dosis pares de dosis que se consideren equivalentes y se comparan directamente por medio de pruebas en duplicado. Los resultados correlacionados para cada par aportan datos para estudio de las diferencias cualitativas entre los distintos productos.
5. Sólo se considerarán equivalentes dosis de dos preparaciones, si producen: (*a*) la misma respuesta media, (*b*) distribución simétrica alrededor de la diagonal de reacciones iguales, y (*c*) una dispersión de dicha diagonal no mayor que la obtenida con dos dosis iguales de una sola preparación.
6. Si los múltiplos de un par de dosis equivalentes resultan siempre equivalentes, puede considerarse entonces a las dos dosis como cualitativamente iguales. La razón constante entre dosis equivalentes es entonces denominada la razón de potencia entre las preparaciones.

Ofrécese una comparación entre dos tuberculinas purificadas: DPP-S de los Estados Unidos y RT XIX-XX-XXI de Dinamarca. Dosis de 0.0002 mg. de cada una resultaron ser equivalentes al ser administradas a escolares vacunados con BCG.

El décuplo de dicha dosis (0.002 mg.) de ambas preparaciones produjo iguales respuestas medias en un grupo mixto de niños vacunados y sin vacunar que no habían reaccionado a la dosis menor de RT. Sin embargo, DPP-S se mostró más potente que RT en los niños no vacunados, sucediendo lo contrario en los vacunados. Había, pues, una leve diferencia cualitativa entre las dos preparaciones, correspondiendo a la causa (BCG) de la sensibilidad en los niños.

En las poblaciones humanas sensibilizadas por factores desconocidos, pueden usarse preparaciones cualitativamente distintas para descubrir la existencia de distintos agentes sensibilizantes.

## RESUME

*Une méthode pour la standardisation des préparations de tuberculine basée sur les réactions intradermiques chez l'homme. Etude comparative de deux tuberculines purifiées*

Description d'une méthode pour la normalisation des préparations de tuberculine selon leur capacité de production de réactions cutanées chez l'homme. Les principales étapes de la méthode sont:

1. Une dose adéquate de la préparation standard est choisie, de préférence elle sera dans les limites des doses classiques utilisées.

2. Différentes doses de préparations inconnues sont comparées au standard à l'aide d'un double test de Mantoux.

3. La dose de préparation inconnue donnant une réaction moyenne semblable au standard est déterminée, celle-ci est dénommée, à titre provisoire, dose équivalente.

4. Des paires de doses considérées équivalentes sont choisies parmi diverses fractions de l'échelle des doses, elles sont comparées directement par des tests doubles. Les résultats correspondants pour chaque paire constituent les données utilisées pour l'étude des différences qualitatives entre les produits.

5. Les doses provenant de deux préparations ne peuvent être considérées comme équivalentes que si elles déterminent (*a*) une même réaction moyenne (*b*) une répartition symétrique autour de la diagonale des réactions égales et (*c*) une dispersion à partir de cette diagonale ne dépassant pas celle obtenue avec deux doses égales d'une préparation.

6. Si des multiples d'une paire de doses équivalentes se montrent toujours équivalents, les deux préparations peuvent alors être considérées qualitativement égales. Le rapport constant entre les doses équivalentes est alors désigné comme le rapport d'activité entre les préparations.

Il a été procédé à une étude comparative de deux tuberculines purifiées, PPD-S des Etats-Unis et RT XIX-XX-XXI du Danemark. Des doses de 0,0002 mg. de chacune des tuberculines administrées à des enfants d'âge scolaire, vaccinés par le BCG, ont été trouvées équivalentes.

Une dose dix fois plus grande que cette dose (0,002 mg.) de l'une et l'autre de ces préparations a produit des réactions moyennes égales dans un groupe mixte composé d'enfants vaccinés et non vaccinés, qui étaient anergiques à la dose plus faible de RT. Cependant, dans le groupe d'enfants non-vaccinés, PPD-S était plus actif que RT, alors que le contraire se produisait dans le groupe vacciné. Une légère différence entre les préparations, correspondant à la source de la sensibilité (BCG) chez les enfants, était ainsi démontrée.

Dans les groupes humains dont l'agent sensibilisant est inconnu, des préparations qualitativement différentes peuvent être utilisées pour mettre en évidence l'existence d'agents sensibilisants différents.

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# THE ISOLATION AND CHEMISTRY OF A PROTEIN FRACTION OF M. TUBERCULOSIS AND ITS ABILITY TO SENSITIZE CELLS[1,2]

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## INTRODUCTION

Numerous attempts have been made to isolate a biologically active protein fraction from the bodies of *Mycobacterium tuberculosis*, mainly with the objective of improving the yield and simplicity of obtaining such a product for skin testing, for serologic tests, or for an immunizing agent in tuberculosis.

During the last fifteen to twenty years there have been a number of studies on this subject. Heidelberger and Menzel (1) used either frozen and vacuum-dried organisms or cells killed by immersion in acetate buffer. After extracting these with acetone, they obtained at least three different antigenic protein fractions by successive extraction with solutions of increasing alkalinity. Grönwall (2) extracted with 0.05 N sodium hydroxide from bacilli (BCG), killed by freeze-drying, and grinding in a ball-mill at −60° to −70°C., a small molecular weight protein with a potency of about the same order as that of standard PPD. Heckly and Watson (3) worked with live bacillary cells ruptured by freezing rapidly at −70°C. in the presence of ether and obtained extracts with phosphate or borate buffers. After removing from these extracts most of the nucleic acid by adsorption to calcium phosphate, they obtained fractions which differed in their relative potencies but which had the same electrophoretic mobility. From this they concluded they were still dealing with a mixture of proteins.

Stacey, Kent, and Nassau (4) extracted moist steam-killed bacilli with solid urea and obtained a polysaccharide complex containing mainly a single electrophoretic component which showed serologic specificity with human tuberculous antisera. Iland, Jones, Baldwin, and Gilbert (5) carried these studies further and extracted living bacilli with urea, isolating a fraction which was mainly protein, 95 per cent homogeneous in electrophoresis, and able to give a positive tuberculin skin reaction and a positive complement fixation test with sera from tuberculous patients. This process of extraction has the advantage over previous methods of being extremely simple and yet giving large yields of the protein.

During the fractionation studies on tuberculin reported previously from this laboratory (6), quantities of bacilli of the H37Rv and H37Ra strains were available. An attempt was made, therefore, to isolate from these bacillary bodies, by the urea extraction process, a protein fraction which could be compared with those obtained from the filtrates. The results on the yields and chemical properties of this fraction and also its biologic potencies are presented. Studies of the bio-

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[2] This study was aided by grants from the Committee on Medical Research of the American Trudeau Society, medical section of the National Tuberculosis Association.

[3] Fellow of the National Tuberculosis Association, 1948–1951.

logic potency of the material include its immunizing capacity, its serologic specificity, and its ability to elicit the allergic and Arthus reactions, as can be demonstrated by the skin reaction and also by the direct effect of the fractions on the cells in tissue cultures. Many of the findings are confirmatory of the results obtained by the English investigators. Comparisons of the data in the present study with the corresponding properties of the filtrate fractions from the previous study (6) will relate the two investigations.

The biologic studies show that a specific cellular sensitization can be induced by repeated injection of this fraction, as well as of some tuberculin fractions, into normal animals and, therefore, that such sensitization is not exclusively dependent upon a state of tuberculous infection, as has been maintained by other investigators.

## Materials and Methods

*Preparation of fractions used:* The two cultures[4] of tubercle bacillus, H37Rv and H37Ra, were grown on Long's synthetic culture medium for eight weeks, after which they were filtered without killing the cells. In each case some of the moist bacilli were mixed with twice their weight of solid urea, as recommended by Stacey, Kent, and Nassau (4), and placed in the incubator at 37°C. for seventy-two hours with frequent stirring. At the end of this period one volume of water was added, and the mixture was then stirred and filtered first through paper and then through a Seitz filter. The solution had a greenish color. It was dialyzed for twenty-four hours against distilled water and then concentrated by ultrafiltration and again dialyzed to free it of urea, and refiltered through the Seitz filter. The fraction made from the H37Rv strain was called *101 B* and that from the H37Ra, *102 B*, and they will be referred to by these numbers hereafter.

The preparation of the A, B, and C fractions from the tuberculin filtrate was described previously (7) and, therefore, only a brief description of them will be given here. The C fraction was precipitated at pH 4.0 with acetic acid while the A and B remained soluble. The A fraction had a lower mobility in electrophoresis, while the B and C were faster and had a mobility similar to that of the *101 B* fraction.

PPD-S was a preparation made from heated culture filtrate and used as a standard for tuberculin testing (8).

*Electrophoretic analyses:* Electrophoretic analyses were made in the Tiselius electrophoresis apparatus, using phosphate buffer pH 7.6 $\mu = 0.1$ for 102 minutes.

*Chemical analyses:* Nucleic acid was determined by the Dische diphenylamine reaction, as previously adapted for quantitative analysis (9); and carbohydrate, by the anthrone method (10), using glucose as a standard. Nitrogen was determined by the micro-Kjeldahl method. Measurements in the ultraviolet region were made in the Beckman spectrophotometer. Amino acid analyses were made by means of the Moore and Stein technique (11) of chromatography on starch columns.

## Biologic Techniques

*Skin reactions:* Skin reactions were induced by intradermal injections of 0.1 ml. amounts, containing 5$\gamma$ of protein, into tuberculous guinea pigs selected for their high degree of sensitivity to tuberculoprotein. The reactions were measured in two diameters at right angles to each other, and only the averages of these two readings were recorded. The second value in the recorded reactions represents the estimated height. Twenty-four-hour readings are listed above the forty-eight-hour readings in each case. No reactions were given

[4] Supplied by Mr. W. Steenken, Jr., of the Trudeau Laboratory, Trudeau, New York.

with any of the fractions in normal guinea pigs. Simultaneous tests were made on each guinea pig with 5γ doses of several different fractions.

*Pyrogenic reactions:* Pyrogenic reactions were determined by giving intravenously to normal rabbits 5 mg. amounts of the respective proteins and following their rectal temperatures at frequent intervals thereafter (12).

*Precipitin reactions:* For the precipitin reactions, 0.1 ml. amounts of the serially diluted antigens were carefully layered over 0.1 ml. volumes of the different anti-sera. The rings of specific precipitate were recorded after two hours' incubation at 37°C., and after this the tubes were shaken and placed in the refrigerator overnight. In the morning the amount of precipitate formed was noted on a scale of 1 to 4 plus, and the last tube showing a definite precipitate was considered to be the titer.

*Sensitization:* Sensitization of the animals for the study of their cells in tissue culture was done as follows:

> Three groups of guinea pigs were given weekly intradermal injections for twenty weeks of one of the following protein fractions; the *101 B* fraction, the A plus B protein fraction (90 I B), or C protein fraction (94 I). Ten milligram doses were given in all cases except with the *101 B* fraction. In this latter case, the first injection of 10 mg. gave a definite reaction and, therefore, the dosage was reduced to 5 mg. for 15 more injections and then to 2.5 mg. for the 6 last injections. More severe reactions followed these small doses of *101 B* than were caused by the 10 mg. doses of the A plus B or C protein fractions. Of the two latter, the C protein always gave the least reactions, which confirms the results in rabbits reported previously (13).

Tissue cultures were prepared as follows:

> At intervals during the series of injections peritoneal exudates, mainly of polymorphonuclear cells, were induced in guinea pigs by intraperitoneal injections of a balanced salt solution. The fluid was removed after twenty-four hours by gravity as described previously (14). The exudates were centrifuged after the addition of the test solution, and plaques were made of the sedimented cells by the addition of homologous plasma. The plaques were then cut into pieces, washed in salt solution, and placed in plasma containing one of the protein fractions dissolved in balanced salt solution to form hanging drop tissue cultures (3 or 4 pieces to a culture) as described previously. The control cultures received balanced salt solution without the test product. After eighteen to twenty hours' incubation at 37°C., the tissue cultures were evaluated for the extent of migration, granulation, agglutination, vacuolization, and disintegration of the cells. The average extents of these various effects on 6 to 9 pieces of tissue in each case were graded from 0 to 8 and recorded under these headings in tables 7 and 8.

When sensitized or tuberculous cells were used, the cells of a normal guinea pig were usually run in parallel as a control of the effect of the product studied on normal cells. The effect produced by a test solution must be considered in comparison with the appearance of the cells from the same animal when these are cultured in the absence of the test substance, since the sensitivity of cells from different animals subjected to the same treatment varies considerably. A reaction is considered to be specific only to the extent that it surpasses that given by the test solution on normal cells.

## Results

### YIELDS

The yields of the urea-extracted fractions obtained are shown in table 1. The solutions were kept frozen at −6°C. in order to prevent the growth of contaminating organisms, as has been the usual custom with all other tuberculin protein fractions. However, on thawing, the *102 B* gelled and became permanently insoluble, preventing complete analyses on this fraction.

## CHEMICAL AND PHYSICAL PROPERTIES

The electrophoretic patterns (figure 1) showed that 85 or 80 per cent of the total *101 B* (H37Rv) or *102 B* (H37Ra) fractions, respectively, consisted of one component which appeared to be nearly homogeneous and with a mobility of

TABLE 1

YIELD OF UREA-EXTRACTED PROTEIN FROM TUBERCLE BACILLI, STRAINS H37 AND H37Ra

| STRAIN OF TUBERCLE BACILLI | LOT NUMBER | UREA-EXTRACTED FRACTION FROM BACILLI | | GRAMS OF PROTEIN ISOLATED FROM FILTRATE PER LITER |
|---|---|---|---|---|
| | | Grams of Moist Bacilli Used | Total Grams of Fraction Obtained | |
| H37Rv | 101 B | 575 | 1.79 | 0.39 |
| H37Ra | 102 B | 614 | 2.20 | 0.36 |

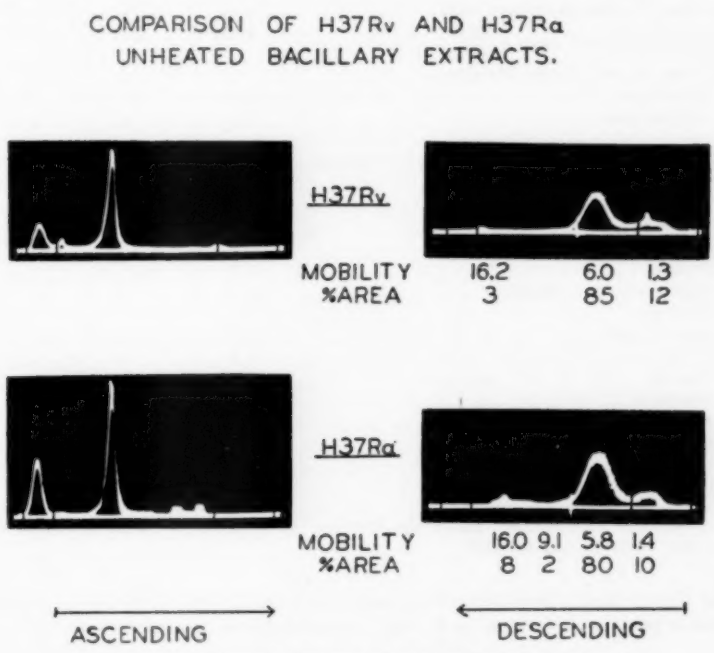


FIG. 1. Electrophoretic diagrams of unheated bacillary extracts

$-6.0$ or $-5.8$ cm.$^2$ volt$^{-1}$sec$^{-1}$. There were very small amounts of components with higher mobilities ($-9.1$ and $14.0$ to $-16.2$), which were probably nucleoprotein or free nucleic acid.

The *101 B* fraction was found to have a nitrogen content of 12.2 per cent on

the dry weight, equivalent to a content of 73.3 per cent protein by calculation. The amino acids found in the *101 B* fraction are shown in table 2. Analysis showed 2.9 per cent nucleic acid and 24.8 per cent carbohydrate. Measurement of the ultraviolet absorption gave a ratio (7) of the densities at $\frac{\lambda\ 280}{\lambda\ 250} = 1.25$. This corresponded closely to the ratio found for a protein, designated C, isolated from

TABLE 2

CHEMICAL ANALYSES OF 101 B AND THE FILTRATE PROTEINS A AND C

| | H37Rv (101 B) | FILTRATE PROTEINS | |
|---|---|---|---|
| | | C | A |
| Per cent of solids as nitrogen | 12.2 | 13.3 | 4.1 |
| Per cent of solids as protein | 73.3 | 83.1 | 25.9 |
| Per cent of solids as nucleic acid | 2.9 | 0.35 | 1.1 |
| Per cent of solids as carbohydrate | 24.8 | 2.7 | 60.8 |
| Ultraviolet density ratio = $\frac{\lambda 280}{\lambda 250}$ | 1.25 | 1.28 | 2.3 |
| Per cent of total as phenolic groups: | | | |
| Hydrolyzed | 4.0 | 3.8 | 7.6 |
| Unhydrolyzed | 3.2 | 3.8 | 4.6 |
| Nitrogen as per cent of total protein nitrogen as: | | * | * |
| Leucine + isoleucine | 10.9 | | |
| Phenylalanine | 0.8 | | |
| Valine | 6.2 | | |
| Methionine + tyrosine | 2.2 | | |
| Proline | 3.7 | | |
| Glutamic acid + alanine | 18.3 | | |
| Threonine | 4.5 | | |
| Aspartic acid | 6.7 | | |
| Serine | 3.8 | | |
| Glycine | 8.6 | | |
| Ammonia | 9.4 | | |
| Arginine | 4.3 | | |
| Lysine | 4.1 | | |
| Histidine | 2.0 | | |
| Cystine | 1.0 | | |

* For these analyses see "Tuberculin Proteins" by F. B. Seibert, Transactions of the Faraday Society, August, 1952.

the filtrate and is in contrast to a ratio of approximately 2.3 found for another filtrate protein, designated as A, and characteristic for this type of protein.

## BIOLOGIC PROPERTIES

*Skin reactions:* The results show (table 3) that the potency of the urea-extracted fraction, as measured by the skin reactions on tuberculous guinea pigs, is somewhat less per milligram of nitrogen than that of the total protein fraction of the tuberculin filtrates, but possibly slightly greater than that of PPD-S.

*Arthus sensitization:* For the development of sensitization and its exhibition by means of the intradermal test, guinea pigs were injected intracutaneously ap-

TABLE 3

COMPARATIVE SKIN REACTIONS OF 101 B, 102 B, PPD-S, AND FILTRATE FRACTIONS IN TUBERCULOUS ANIMALS

| NUMBER OF GUINEA PIGS | AVERAGE SKIN REACTION DIMENSIONS IN MILLIMETERS WITH 5γ DOSES OF PROTEIN* IN: | | |
|---|---|---|---|
| | Urea-Extracted Protein (101 B) | Total Filtrate Protein (101) | PPD-S |
| 9 | 20 × 2.5<br>19 × 2.9 | 23 × 3.0<br>21 × 3.1 | 19 × 2.5<br>15 × 2.3 |
| 6 | 19 × 3.1<br>21 × 3.3 | 22 × 3.1<br>22 × 3.3 | 19 × 2.5<br>19 × 3.0 |
| | (102 B) | (102) | PPD-S |
| 8 | 19 × 2.5<br>18 × 2.3 | 21 × 2.5<br>21 × 2.3 | 19 × 2.5<br>17 × 2.3 |

* These doses were based on the protein calculated from the nitrogen contents.

TABLE 4

PYROGENIC EFFECT OF FRACTIONS

| PRODUCTS | | DEGREES CENTIGRADE RISE IN TEMPERATURE |
|---|---|---|
| Polysaccharide I | 870 | 0° |
| Polysaccharide II | 100 V<br>85<br>96 A IIIa | −0.1°<br>1.0°<br>0.5° |
| Protein A | 100 II<br>100 IV<br>100 III | 0°<br>−0.25°<br>0.45° |
| Protein B | 97 B<br>96 A III b | 1.6°<br>2.0° |
| Protein C | 94 I<br>96 A I | 1.0°, 1.0°, 0.6°, 0.95°<br>1.85°, 0.85° |
| Urea-extracted protein | 101 B | 0.7°, 0.6°, 0.3°, 1.2°, 1.15°, 0.6°<br>0.6°, 1.2°, 0.7°, 1.4° |
| Distilled water | | −0.2° |
| Buffer number 2 | | −0.3° |

proximately once a week. It was pointed out previously (13) that the A type of tuberculin protein elicited a much more severe Arthus reaction in rabbits than did the C type of protein. In this study also, the same relationship was true in

guinea pigs. For example, only four injections of 10 mg. of an A plus B protein were required to produce a necrotic reaction, whereas ten injections of a C-type protein were necessary and, even with this, only large diffuse edematous reactions appeared. The urea-extracted protein (*101 B*) gave the most severe reactions, much more localized and with deep necrotic centers. On the second injection, severe reactions appeared and, even on the first injection, a definite reaction occurred. In the case of the series of guinea pigs being sensitized for the tissue culture studies, it was found necessary to reduce the dosage of the *101 B* to 5 mg. per injection after the first injection and even to 2.5 mg. after fourteen injections.

*Pyrogenic reactions:* No fever followed the injection of three different tuberculin protein fractions of the A type, as shown in table 4. The B- and C-type tuberculin

TABLE 5

PRECIPITIN TITERS WITH DIFFERENT ANTIGENS

| ANTISERA TO: | ANTIGENS | | |
|---|---|---|---|
| | A | C | 101 B |
| A | 1,000,000 (s) | 100,000 (s) | 25,000 (s) |
| | 2,500,000 (s) | 250,000 (s) | 100,000 (s) |
| C | 25,000 (w) | 250,000 (s) | 50,000 (s) |
| | 10,000 (w) | 100,000 (s) | 100,000 (w) |
| | 1,000 (w) | 500,000 (s) | 50,000 (w) |
| 101 B | 1,000 (w) | 50,000 (s) | 25,000 (s) |
| | 5,000 (w) | 500,000 (s) | 100,000 (s) |
| BCG | 500,000 (w) | 100,000 (s) | 250,000 (s) |
| | 250,000 (w) | 25,000 (s) | 50,000 (w) |

(s) = heavy precipitate.
(w) = weak precipitate.

proteins and also the urea-extracted protein (*101 B*) were all followed by febrile reactions. All of these three latter fractions had similar mobilities in electrophoresis which were higher than those found for the A type of proteins. It is highly probable that the B and C proteins are true pyrogens, since they were isolated from the same solutions in which non-pyrogenic polysaccharides and A proteins were also present. Most of the polysaccharide fractions tested gave no fever, but the less pure one (Lot 85) did. As was pointed out (15), however, since strictly rigid technique for the preparation of non-pyrogens was not employed during the entire growth of the organisms and the isolation procedures, a definite conclusion on pyrogenicity cannot be made.

*Precipitin reactions:* The specificity of the antigens was determined by means of the precipitin reaction. Table 5 shows that definite specificity was noted for the A and C proteins, and that the *101 B* protein crossed better with the C protein than it did with the A protein. This corresponds with the fact that chemically

the *101 B* and C resembled each other. The BCG antisera gave the highest titers with the A antigen, as was true in a former experiment (16).

### *Immunity*

In view of the fact that no immunity was demonstrated (13) in rabbits sensitized either with the A or the C tuberculin proteins from the filtrate, it was of interest to determine whether this fraction (*101 B*), which was extracted from

TABLE 6

DAYS OF LONGEVITY FOLLOWING INOCULATION AND DEGREE OF TUBERCULOSIS IN GUINEA PIGS PREVIOUSLY SENSITIZED WITH NOTHING, BCG, AND 101 B

| NOTHING | | BCG | | 101 B | |
|---|---|---|---|---|---|
| Days | Degree of Tuberculosis | Days | Degree of Tuberculosis | Days | Degree of Tuberculosis |
| 42 | 0 | 59 | 0 | 64 | ++ |
| 150 | +++ | 62 | 0 | 83 | ++++ |
| 165 | ++++ | 178 | 0 | 94 | +++ |
| 195 | + | 178 | 0 | 157 | ++ |
| 262 | ++++ | 489 | 0 | 185 | ++ |
| 274 | ++++ | 532+ | | 268 | 0 |
| 274 | +++ | 532+ | | 279 | ++++ |
| 279 | ? | 532+ | | 285 | ++++ |
| 293 | +++ | 532+ | | 302 | ++++ |
| 357 | + | 532+ | | 321 | ++ |
| 447 | ++++ | 532+ | | 332 | +++ |
| 532+ | | | | 422 | +++ |
| | | | | 493 | +++ |
| Average 273+ | | 422+ | | 253 | |

the bacillary bodies, might be a better immunizing agent. The following experiment was, therefore, undertaken.

Twelve guinea pigs were used in each group. *Group A* had no previous treatment; *Group B* had been sensitized by weekly intradermal injections of 2.5 mg. of *101 B*; and *Group C* had been sensitized by 4 intradermal injections (2.5, 1.0, 0.5, and 0.25 mg.) of BCG during ten weeks prior to inoculation. All guinea pigs were then inoculated subcutaneously in the groin with 0.00033 mg. of moist bacilli (strain H37Rv).

From the results given in table 6, it is obvious that a definite immunity was effected in the group vaccinated with BCG. On the other hand, no increase in longevity, in comparison with the controls, was found in the group sensitized with the *101 B* fraction, and, with one exception, those that died all showed evidence of gross tuberculosis. It is safe, therefore, to conclude that the *101 B* fraction does not induce immunity.

### *Tissue Cultures*

*Effect of 101 B on tuberculous cells:* Table 7 and figure 2 (left) show that the *101 B* fraction caused a marked inhibition to migration of the leukocytes from

## TABLE 7

### Reactions of Leukocytes to 101 B Fraction

| Mg. of 101 B in test solution | Extent of reaction on leukocytes from guinea pigs | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | | | | Tuberculous | | | | Sensitized | | | | | |
| | Migration | Granulation | Agglutination | Vacuolization | Migration | Granulation | Agglutination | Vacuolization | Migration | Granulation | Agglutination | Vacuolization | Disintegration | |
| 0 | 4 | 3 | 2-3 | | 7-8 | 2 | 2-3 | | | | | | | |
| 0.1 | 4-5 | 3 | 3-4 | | 5 | 3 | 4 | | | | | | | |
| 0.2 | 4-5 | 3 | 4 | | 3 | 3 | 4 | | | | | | | |
| 0 | 4 | 3 | 2 | | 4 | 2-3 | 4-5 | | | | | | | |
| 0.2 | | | | | 1-2 | 3 | 4-5 | 1 | | | | | | |
| 0 | 6 | 3 | 3-4 | | 5-6 | 3 | 3-4 | | | | | | | |
| 0.17 | 4-5 | 3 | 3-4 | | 2-3 | 3 | 4 | 1 | | | | | | |
| 0.2 | | | | | 0-1 | 3-4 | 1 | | | | | | | |
| | | | | | BCG | | | | | | | | | |
| 0 | | | | | 7 | 3 | 3 | | | | | | | |
| 0.17 | | | | | 4-5 | 3 | 3-4 | | | | | | | |
| 0.2 | | | | | 4 | 3 | 3 | | | | | | | |
| 0 | 7-8 | 3 | 2 | | | | | | 7-8 | 3 | 3 | | | |
| 0.2 | 7 | 3 | 3 | 0-1 | | | | | 6 | 3 | 4 | 1 | | |
| 0 | 6 | 3 | 3 | | | | | | 5-6 | 3 | 2 | | | |
| 0.2 | 3-4 | 3 | 4 | | | | | | 2 | 3 | 4-5 | 1 | 1 | |
| 0 | | | | | | | | | 5-6 | 3 | 3-4 | | | |
| 0.1 | | | | | | | | | 5-6 | 3 | 3-4 | | | |
| 0 | | | | | | | | | 7-8 | 3 | 2 | | | |
| 0.2 | | | | | | | | | 4 | 3 | 4 | 1 | | |
| 0 | | | | | | | | | 7-8 | 2 | 3 | 1 | | |
| 0.1 | | | | | | | | | 7 | 3 | 4-5 | 1 | | |
| 0.17 | | | | | | | | | 6 | 3 | 4-5 | 1 | | |
| 0 | 3-4 | 3 | 3 | | | | | | 6-7 | 3 | 3 | | | |
| 0.17 | 3 | 3 | 3 | | | | | | 5 | 3 | 4 | 0-1 | | |
| 0.2 | 2-3 | 3 | 3 | | | | | | 4 | 3 | 4 | 1 | | |
| 0 | | | | | | | | | 6 | 3 | 3 | | | |
| 0.17 | | | | | | | | | 5 | 3 | 3-4 | 0-1 | | |
| 0.2 | | | | | | | | | 5-6 | 3 | 3-4 | 0-1 | | |
| 0 | 6 | 3 | 3-4 | | | | | | 7-8 | 3 | 3 | | | |
| 0.17 | | | | | | | | | 5 | 3 | 4 | 0-1 | | |
| 0.2 | | | | | | | | | 4 | 3 | 4 | 0-1 | | |

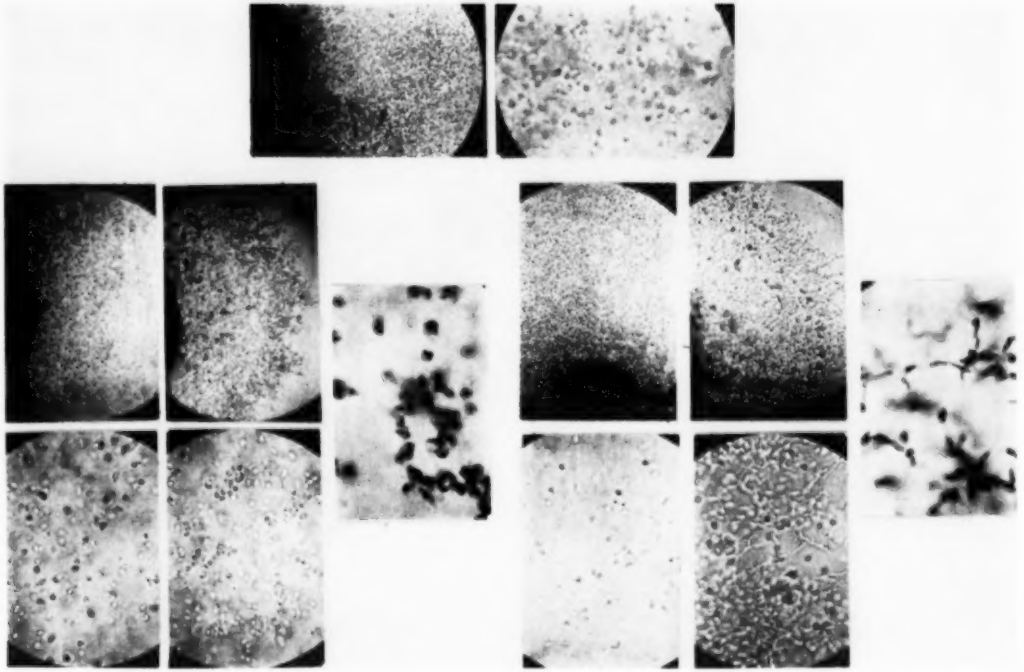

FIG. 2. Effect of tubercle bacillary protein (*101 B*, 0.2 mg. per ml.) on normal, tuberculous, and sensitized guinea pig leukocytes in living tissue cultures (18 to 20 hours).

Top—*Normal cells* plus balanced salt solution: (left) low power; (right) high power.

Left lower group of five pictures—*Tuberculous cells:* (left) low and high power of tuberculous cells plus balanced salt solution; (right) low and high power of tuberculous cells plus *101 B*, showing inhibition to migration and clumping of cells; (single picture) oil immersion picture of clumped tuberculous cells plus *101 B*.

Right lower group of five pictures—*Sensitized cells:* (left) low and high power of sensitized cells plus balanced salt solution; (right) low and high power of sensitized cells plus *101 B*, showing inhibition to migration and clumped and extended cells; (single picture) oil immersion picture of extended cells.

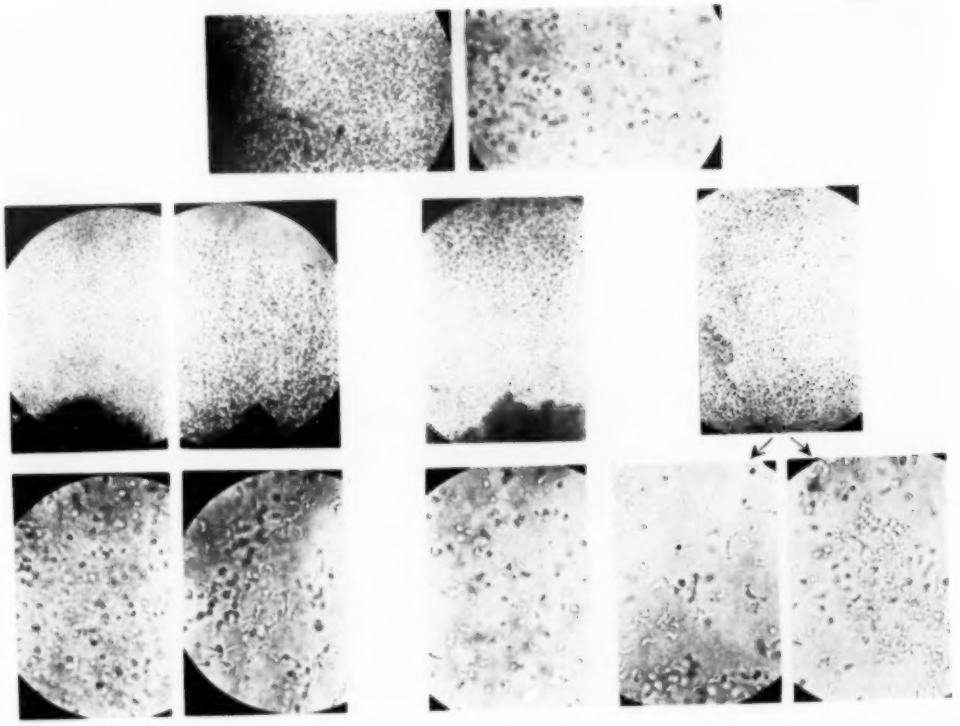

FIG. 3. Effect of tubercle bacillary protein (*101 B*, 0.2 mg. per ml.) on normal and sensitized guinea pig leukocytes in living tissue cultures (18 to 20 hours) in two more experiments.

Top—*Normal cells* plus balanced salt solution: (left) low power; (right) high power.

Left group of four pictures—*Sensitized cells:* (left) low and high power of sensitized cells plus balanced salt solution; (right) low and high power of sensitized cells plus *101 B*, showing inhibition of migration and stretching of cells.

Right group of five pictures—*Sensitized cells:* (left) low and high power of sensitized cells plus balanced salt solution; (right) low power (above) of sensitized cells plus *101 B*, showing inhibition of migration; (lower left) high power, showing stretching of cells; (lower right) showing clumping of cells.

## TABLE 8

### Reactions of Leukocytes to A Plus B Protein and to C Protein

| Mg. in test solution | Extent of reaction on leukocytes from guinea pigs | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Normal | | | | Sensitized | | | |
| | Migration | Granulation | Agglutination | Vacuolization | Migration | Granulation | Agglutination | Vacuolization |
| A plus B protein | | | | | | | | |
| 0 | 4–5 | 3 | 2 | | 4 | 3 | 2 | |
| 0.1 | 4 | 3 | 3 | | 3 | 3 | 3–4 | |
| 0 | 7–8 | 3 | 2 | | | | | |
| 0.1 | 7–8 | 3 | 3 | | | | | |
| 0.17 | 7 | 3 | 3 | | | | | |
| 0 | 7–8 | 3 | 3 | | | | | |
| 0.1 | 7–8 | 3 | 4 | | | | | |
| 0 | 7–8 | 3 | 3 | | | | | |
| 0.1 | 7–8 | 3 | 4 | | | | | |
| 0 | | | | | 6 | 3 | 3 | |
| 0.1 | | | | | 4 | 3 | 3–4 | |
| 0 | | | | | 4 | 3 | 3 | |
| 0.1 | | | | | 2–3 | 3 | 4–5 | |
| 0 | 7–8 | 3 | 3 | | 7–8 | 3 | 3 | |
| 0.17 | 7–8 | 3 | 3–4 | | 4–6 | 3 | 4 | 1 |
| 0.2 | 3 | 3 | 4 | 1 | 2 | 3 | 4–5 | 1 |
| 0 | 7 | 3 | 3 | | 7–8 | 3 | 3 | |
| 0.1 | 6 | 3 | 3 | | 5 | 3 | 4–5 | |
| 0.2 | 5–6 | 3 | 3 | 1 | 4 | 3 | 4–5 | 1 |
| C protein | | | | | | | | |
| 0 | | | | | 6–7 | 3 | 2 | |
| 0.2 | | | | | 3 | 3 | 4 | |
| 0 | 4 | 3 | 2 | 1 | 4–5 | 3 | 2 | 1 |
| 0.2 | 3 | 3 | 3 | 1 | 4 | 3 | 2 | 1 |
| 0 | 7 | 3 | 3 | | 4 | 3 | 2 | |
| 0.17 | 6–7 | 3 | 3–4 | | 2–3 | 3 | 4 | |
| 0.2 | 4–5 | 3 | 4 | | 2 | 3 | 4 | |
| 0 | 7–8 | 3 | 3 | | 7–8 | 3 | 3 | |
| 0.17 | 7–8 | 3 | 3 | | 6–7 | 3 | 4–5 | |
| 0.2 | 7–8 | 3 | 3 | | 6–7 | 3 | 4 | |

TABLE 8—*Continued*

EXTENT OF REACTION ON LEUKOCYTES FROM GUINEA PIGS

| MG. IN TEST SOLUTION | Normal | | | | Sensitized | | | |
|---|---|---|---|---|---|---|---|---|
| | Migration | Granulation | Agglutination | Vacuolization | Migration | Granulation | Agglutination | Vacuolization |
| C protein (*Cont.*) | | | | | | | | |
| 0 | 7 | 3 | 2 | | | | | |
| 0.2 | 6 | 3 | 4 | | | | | |
| 0 | 7 | 3 | 3 | | | | | |
| 0.2 | 6 | 3 | 4 | | | | | |
| 0 | 4-7 | 3 | 3 | | 6 | 3 | 4 | |
| 0.17 | 7 | 3 | 4 | | 7-8 | 3 | 4 | |
| 0.2 | 4 | 3 | 4 | | 7 | 3 | 4 | |
| 0 | 7 | 3 | 3 | | 7-8 | 3 | 3 | |
| 0.17 | 5-6 | 3 | 4-5 | | 7 | 3 | 4-5 | |
| 0.2 | 4 | 3 | 4-5 | | 6 | 3 | 4-5 | |
| 0 | 4-5 | 3 | 4 | | 7 | 3 | 4 | |
| 0.17 | 7 | 3 | 4 | | 6 | 3 | 4-5 | 1 |
| 0.2 | 6 | 3 | 4-5 | | 4-5 | 3 | 4-5 | 1 |
| 0 | 7 | 3 | 3 | | 6-7 | 3 | 3 | |
| 0.17 | 7 | 3 | 3 | | 6-7 | 3 | 3-4 | |
| 0.2 | 7 | 3 | 3-4 | | 6-7 | 3 | 3-4 | |

tuberculous guinea pigs in tissue cultures; also, a definite clumping and even vacuolization of the cells in two experiments. The cells from a guinea pig sensitized with BCG in one experiment were also inhibited in migration by the *101 B*, possibly to a less extent than those from the tuberculous animals. In this case, there was also a tendency toward alignment of the cells, as had been noted previously (14) in connection with the action of A protein on tuberculous cells in tissue cultures.

In some instances a certain degree of clumping of normal cells and even a slight inhibition to migration also occurred, but only with the higher concentration of *101 B*, showing that the differential between specific and nonspecific reactions was relatively small, as pointed out previously (14).

*Effects of 101 B on sensitized cells:* The migration of cells of uninfected animals, which had been sensitized by repeated injections of the *101 B* fraction, was also inhibited when they were placed in contact with this fraction, as shown in table 7 and figure 2 (right) and figure 3. The degree of inhibition was possibly somewhat less extensive than that of the tuberculous cells, but in 7 of 8 experiments definite clumping of the cells and frequent increased vacuolization took place; in one experiment, even disintegration of the cells was observed. Therefore, there

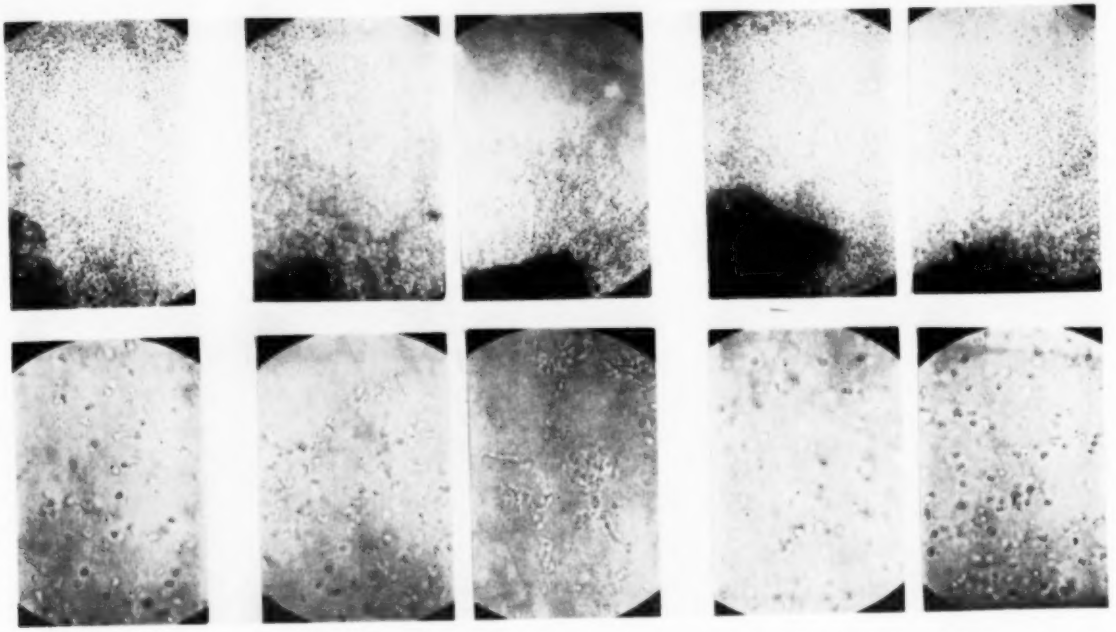

FIG. 4. Effect of tuberculin protein (A plus B, 0.17 and 0.2 mg. per ml.) on normal and sensitized guinea pig leukocytes in living tissue cultures (18 to 20 hours).

Left group of pictures—*Sensitized cells* plus balanced salt solution; low power above; high power below.

Middle group of four pictures—*Sensitized cells* plus A and B protein: (top pictures) low power; (bottom pictures) high power; (left) sensitized cells plus 0.17 mg. of A plus B per ml.; (right) sensitized cells plus 0.2 mg. of A plus B per ml., showing alignment of cells.

Right group of four pictures; *normal cells* plus A and B protein: (top pictures) low power; (bottom pictures) high power (left) cells plus 0.17 mg. of A plus B per ml.; (right) cells plus 0.2 mg. of A plus B per ml.

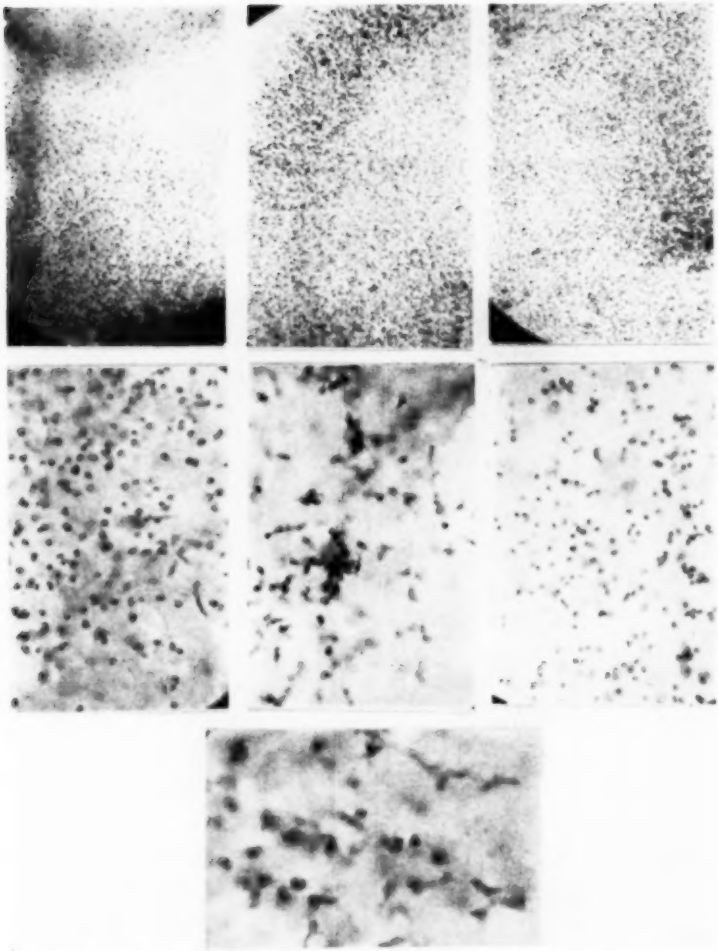

FIG. 5. Effect of tuberculin protein (C', 0.17 mg. per ml.) on normal and sensitized guinea pig leukocytes in living tissue cultures (18 to 20 hours).

Left—*Sensitized cells* plus balanced salt solution: low power above and high power below.

Middle—*Sensitized cells* plus C protein: (top) low power, showing limited migration and area of liquefaction; (center) high power, showing stretching of cells; (bottom) oil immersion power of stretched cells.

Right—*Normal cells* plus C protein: (top) low power; (bottom) high power.

was definite evidence that the cells were specifically sensitive to the *101 B* fraction in the same manner as tuberculous cells. In addition to this, however, these cells displayed a further reaction not conspicuous among the tuberculous cells; namely, a marked elongation and twisting of the polymorphonuclears, as shown in figures 2 and 3, especially at the periphery of the cultures. A careful study of the sensitized cells in media free from test protein revealed a slight tendency toward this phenomenon in the sensitized cells themselves, but the marked effect noted when the sensitized cells and the specific antigen, *101 B*, were mixed was definite evidence that the reaction was highly specific.

The question arises: Are these specific reactions characteristic only of the *101 B* fraction, a bacillary extract, or are the tuberculin proteins also capable of giving rise to specific sensitization of the cells? The following experiments were carried out to answer this question.

*Effect of the A plus B protein on sensitized cells:* There was some inhibition by the A plus B protein to migration of the cells from guinea pigs sensitized with the A plus B fraction, which must be considered specific, since, when the lower concentration of antigen (0.1 mg.) was used, no or very little effect was noted on normal cells (table 8 and figure 4). A large dose, however, had considerable effect on normal cells as well. It is possible that the presence of the B protein in this fraction may account for its increased nonspecific toxicity, since in a previous study (14) it was suggested that the B protein had a relatively great primary toxicity.

Less clumping or stretching of these sensitized cells was noted than with the *101 B*, but instead there was a tendency to flatten out, as was previously (14) noted with tuberculous cells exposed to the A protein.

*Effect of the C protein on sensitized cells:* The inhibition to migration or injury of the cells from animals sensitized with the C protein when exposed to the C protein was only slightly more than that observed with normal cells under the influence of this protein (table 8). There was, however, considerable evidence of a specific stretching of the cells, as noted with the *101 B* sensitized cells (figure 5). In addition, these cultures frequently exhibited areas of liquefaction of the fibrin clot.

## Discussion

In this study it has been shown that the product obtained by urea extraction of the bacillary bodies of tubercle bacilli consisted mainly of one electrophoretic component, the mobility of which corresponded closely with that of the C protein of the filtrate. It further resembled this culture filtrate C protein in its ultraviolet absorption characteristics, shown by its density ratio of 1.25 at 2,800 A to 2,500 A, and in the fact that it precipitated at a pH of approximately 4.0. It is highly significant that only one component with approximately this same mobility could be isolated from the bacillary bodies by Heckly and Watson, using entirely different methods.

The potency of the urea-extracted material as determined by the skin reaction in tuberculous animals, however, was found to be greater than that of the C pro-

tein fractions. It was also more highly antigenic in eliciting the Arthus reaction in normal animals and even was quite toxic on the initial intradermal reaction with a sensitizing dose (10 mg.). As with the A and C proteins, however, it was not capable of inducing in guinea pigs an immunity to challenge with tubercle bacilli.

The effects produced by this urea-extracted protein fraction when put directly in contact with tuberculous cells in tissue cultures demonstrated that its potency for specifically inhibiting the migration of the cells and in causing them to clump was similar to those (14) caused by Old Tuberculin, PPD-S, and the C-type protein. In these respects it resembled the C- more than the A-type protein, and this is consistent with the fact that most of its chemical and physicochemical properties were more like those of the C protein.

The results of the present study demonstrate, moreover, that cells, not only from animals sensitized by infection with tubercle bacilli, but also those sensitized by repeated injections of the proteins from the bacillary bodies and from the filtrate will exhibit specific reactions when placed in direct contact with the specific antigen. The cells of animals rendered sensitive, as demonstrated by a strong Arthus reaction, in contrast to the results of Aronson (17), and Buckley, Buckley, and Gey (18), using horse serum as an antigen, were inhibited in their migration and were clumped by the specific antigen in the same way but only to a slightly less degree than were tuberculous cells. But even more interesting than these effects, the cells were conspicuously elongated and twisted, giving the impression that they were more sticky and active than normal cells. Since these effects appeared mainly in the sensitive cells, it is evident that they were specifically sensitized. Whether this elongation type of cellular sensitization represents merely a stage in the development of sensitization or an entirely different type of sensitization remains to be determined.

All of these reactions were obtained, not only with the protein fraction extracted by urea from the bacillary bodies, but also with the C type of protein from the filtrate but to a less degree. This is consistent with the finding that it was never possible to elicit as severe an Arthus reaction with the C protein as with a corresponding number of injections of the bacillary urea-extracted protein.

Sensitization with the A plus B type of protein also yielded cells whose migration was specifically inhibited to some extent in cultures containing this fraction, but the cells did not elongate and twist as did cells sensitized with the urea-extracted fraction (*101 B*) in the presence of this homologous extracted fraction. Instead, they seemed to align themselves into strands similar to the behavior of tuberculous cells when exposed to the A protein.

The same type of cellular sensitization can, therefore, apparently be developed in animals by either bacterial infection or by repeated injections with certain proteins from tuberculin or from the bacillary bodies. The additional manifestation of activity of the cells (stretching, et cetera) in the Arthus type of sensitization may account for the differences between this type and the allergic type of sensitization which have been emphasized. Moreover, the cells of animals with the Arthus type of sensitization would be acting *in vivo* in the presence of large

amounts of circulating antibodies, whereas those from tuberculous anintals would be acting in the absence of such demonstrable antibodies.

Support for the idea that, not only tuberculin proteins may be able to induce such cellular sensitization, but that perhaps proteins in general may have such potentialities comes from the recent work of Ebert and collaborators. Ebert, Ahern, and Bloch (19) observed *in vivo* in the tuberculous rabbit sticking of the leukocytes to vascular endothelium and such an extensive aggregation of cells that stasis and localized thrombosis occurred in the small venules. Recently, Ebert and Wissler (20) reported "sticking" of white cells to endothelial surfaces four to seven days after the first injection of large amounts of horse serum and similar but greatly intensified reactions following the second injection.

Lucké, Strumia, Mudd, McCutcheon, and Mudd (21) showed that phagocytosis of particles by polymorphonuclears and macrophages was enhanced in the presence of immune serum, and, therefore, in sensitized or infected animals the tubercle bacilli would be more readily phagocytosed. With increased activity and "stickiness" of the cells, as described in the present paper, there would, therefore, be more widespread dissemination of the bacteria, which may or may not be destroyed within them, depending upon the virulence of the organism (22) and the resistance of the host (23). Moreover, the more virulent the organism, the more probable it is that the cell itself may be damaged (24). The mononuclears of these allergic animals are also stimulated to increased phagocytic activity (25) and can, therefore, engulf large numbers of the polymorphonuclears containing tubercle bacilli, as shown by Vorwald (26). Then extensive tubercle formation and subsequent caseation follow.

With the consequent disappearance of polymorphonuclears, due to sticking to the endothelial walls, clumping, and disintegration of the cells under the influence of the protein products of the tubercle bacilli and to phagocytosis by the mononuclears, these latter cells would predominate in the later stages of tuberculosis, as found by Sabin (27), Lurie (25), and others. Moreover, Vorwald (28) has shown that the persistence of polymorphonuclear leukocytes throughout all periods of infection was associated with lowered resistance.

A possible explanation is, therefore, at hand of why previous sensitization to the proteins of the tubercle bacillus predisposes to more rapid and widespread infection and caseation, especially when such animals are infected with large doses of tubercle bacilli (29, 13).

## Summary

A protein fraction was extracted from the bodies of the tubercle bacillus by means of solid urea which had an electrophoretic mobility similar to that of the C protein isolated from tuberculin filtrates and also resembled it chemically and immunologically in the precipitin reaction.

The fraction had a greater potency in eliciting the skin reaction in tuberculous animals than did the C and also gave rise to more severe Arthus reactions. In these respects it more closely resembled the A type of tuberculin protein. Like the C protein it was pyrogenic to the normal rabbit, but had no immunizing power.

The urea-extracted fraction caused specific inhibition to the migration and also clumping and disintegration of polymorphonuclear cells from tuberculous guinea pigs in tissue cultures, similar to that reported previously for the C protein.

Polymorphonuclear leukocytes of animals uninfected but sensitized by repeated injection of this urea-extracted protein were specifically sensitized, since they reacted to it, not only by being inhibited in migration and in clumping, but also were much elongated and twisted and appeared to be sticky. Similar but less intense reactions were elicited by the C protein in animals uninfected but sensitized with this protein.


## Sumario

*El Aislamiento y la Química de una Fracción Proteínica del Bacilo Tuberculoso y Capacidad de la Misma pra Sensibilizar las Células*

De los cuerpos de bacilos tuberculosos extrájose, por medio de urea sólida, una fracción proteínica dotada de una movilidad electroforética semejante a la de la proteína C aislada de filtrados tuberculínicos y parecida también, química e inmunológicamente a la misma, en su precipitinorreacción.

Dicha fracción mostró mayor potencia que la C en la evocación de cutirreacciones e igualmente produjo reacciones de Arthur más intensas. En esos sentidos, se asemejó más a la forma A de proteína tuberculínica. Lo mismo que la proteína C, resultó pirógena para el conejo normal, pero no poseía facultad inmunizante.

Ocasionó inhibición específica de la migración y además de la agrumación y la desintegración de las células polimorfonucleares de cobayos tuberculosos, en los cultivos de tejidos, en forma semejante a la ya descrita para la proteína C.

Además, los leucocitos polimorfonucleares de animales indemnes, pero sensibilizados por inyecciones repetidas de dicha proteína extraída con urea, quedaron sensibilizados específicamente, pues reaccionaron a la misma, no sólo en la inhibición de su migración y agrumación, sino también por quedar muy alargados y retorcidos y parecer pegajosos.



## Resume

*Isolement et caractère chimique de la fraction protéique du bacille tuberculeux et son pouvoir de sensibilisation sur les cellules*

Une fraction protéique a été extraite du corps du bacille tuberculeux à l'aide d'urée à l'état solide, dont la mobilité électrophorétique était semblable à celle de la protéine C isolée de filtrats de tuberculine, et à laquelle elle ressemblait du point de vue chimique et immunologique dans la réaction de la précipitine.

L'activité de cette fraction pour provoquer la réaction cutanée était plus élevée que celle de la protéine C; les réactions d'Arthus provoquées étaient également plus sévères. A cet égard, elle ressemblait plus étroitement au type A de la protéine tuberculeuse. De même que la protéine C, elle se montrait pyrogène pour le lapin normal, mais elle était dépourvue du pouvoir immunisant.

Cette fraction protéique déterminait dans les cultures cellulaires l'inhibition spécifique de la migration ainsi que l'agglutination et la désintégration des cellules polymorphonucléaires des cobayes tuberculeux, comme celà a été précédemment rapporté pour la protéine C.

De plus, les leucocytes polymorphonucléaires d'animaux non infectés, mais sensibilisés par l'injection répétée de cette protéine extraite par l'urée, étaient spécifiquement sensibilisés, étant donné qu'ils y réagissaient, non seulement par l'inhibition de leur migration et de leur agglutination, mais encore ils étaient beaucoup plus allongés et tordus et paraissaient gluants.

*Acknowledgment*

We wish to express our thanks to Dr. Margaret Reed Lewis for her help and counsel in the tissue culture studies.

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# STUDIES OF A PURIFIED TUBERCULIN FRACTION IN TESTING BCG-VACCINATED SUBJECTS[1]

## A Preliminary Study

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### INTRODUCTION

The appearance of tuberculin sensitivity in BCG-vaccinated subjects is the sole clinical sign accepted as a means for judging a successful vaccination. If tuberculin sensitivity fails to occur within eight to twelve weeks after vaccination, it is recommended that revaccination should take place until a positive tuberculin reaction is elicited. Approximately 95 per cent of parenterally vaccinated subjects react to 0.1 to 1.0 "mg." of Old Tuberculin with 6 by 6 mm. or more of induration from forty-eight to seventy-two hours after the intradermal inoculation when tested two to three months after vaccination. It is essential, therefore, that any other antigen used for tuberculin testing of BCG-vaccinated subjects should elicit the same specific sensitivity as Old Tuberculin. It is well known that the degree of tuberculin sensitivity produced by a successful BCG vaccination is, at least within approximately three months after vaccination, considerably less than it is after a spontaneous infection with virulent tubercle bacilli, and that only the lower dilutions of Old Tuberculin will elicit the required minimum of 6 by 6 mm. or more of induration in vaccinated subjects. It is still debatable whether the disparity in degree of tuberculin sensitivity in BCG-vaccinated subjects and in spontaneously infected persons is referable to the avirulence or virulence of the infecting organisms or to the intradermal mode of inoculation in BCG-vaccinated subjects and the mainly intrapulmonary route of infection in spontaneously infected persons. The fact remains that a marked difference in allergy exists in persons inoculated with BCG or infected with virulent tubercle bacilli.

In tuberculosis diagnostic and epidemiologic work, two types of tuberculin are in universal use, namely, Old Tuberculin (OT) and purified protein derivative (PPD). The latter consists of the mixed proteins of the heated culture filtrates, essentially free of inactive substances such as nucleic acid and polysaccharide (1). The dried material remains fairly constant in tuberculin potency under protracted storage conditions, while in diluted form it deteriorates rapidly. On the basis of several thousand tests in human subjects, a first-strength dose of 0.00002 mg. PPD detected almost all highly sensitive cases, without danger of excessively severe reactions; and the second-strength dose of 0.005 mg. PPD detected the remainder of positive cases, that is, persons whose tuberculin sensitivity responds to the conventional standard doses of 0.01 to 1.0 "mg." of OT (2).

[1] From the Division of Laboratories and Research, New York State Department of Health, Albany, New York.

When BCG vaccination was officially approved by the New York State Department of Health in May, 1948 (3), on the recommendation of the Division of Tuberculosis Control, the Division of Laboratories and Research, and the Committee on Public Health and Education of the Medical Society of the State of New York (4), the rules stipulated an optional use of either OT or PPD as test material before and after vaccination, with preference for the two-test procedure; or, if only one test was used, an intermediate dose of either 0.1 "mg." of OT or 0.0002 mg. of PPD. It was recommended that readings should be made at forty-eight to seventy-two hours after the test is performed, and a positive reaction should present definite induration of at least 6 by 6 mm., regardless of whether redness was present or not. If the tuberculin test gave less than the required minimum reaction, it was recommended to revaccinate until a positive tuberculin reaction was elicited. The optional employment of either OT or PPD was agreed upon, since the National Tuberculosis Association had recommended that PPD supersede OT because the former is more amenable to accurate specific protein dosage than the latter (5).

As the present investigators had had no previous experience with PPD in testing BCG-vaccinated subjects, simultaneous tests with OT and PPD before and after vaccination were made in order to compare their equivalence. The same location (midvolar surface of the left and right forearms) was used for all intradermal injections, using alternate arms for OT and PPD on every other subject in order to avoid possible differences in reactivity of the left and right arms. Scrupulously clean new syringes and 26-gauge intradermal point needles were used separately for each freshly prepared tuberculin dilution, and readings were made after forty-eight hours for practical purposes. Vaccination of negative reactors to 1.0 "mg." of OT and 0.005 mg. of PPD was performed with freshly prepared 20 mg. of BCG vaccine per ml. and the multiple puncture technique (6). The effect of this route of vaccination is readily assessed since the resulting vaccination papules leave no doubt about the success of vaccination.

It was soon learned that a considerable discrepancy existed between OT and PPD in successfully vaccinated subjects tested simultaneously eight to twelve weeks after vaccination. Approximately 50 per cent of subjects tested with 0.01 "mg." of OT presented definitely indurated skin reactions measuring 6 by 6 mm. or more in diameter, and approximately 20 per cent of subjects tested with 0.00002 mg. of PPD presented less definitely indurated skin reactions measuring 6 by 6 mm. or more in diameter. Approximately 100 per cent of vaccinated subjects reacted to 1.0 "mg." of OT with 2 and 3 plus indurated skin reactions, and approximately 60 per cent reacted to 0.005 mg. of PPD with mostly 1 and 2 plus indurated skin reactions. Pseudoreactions, faint redness without induration reaching its maximum intensity after twenty-four hours and fading out almost completely within forty-eight hours, were slightly more frequent with the second-strength doses of OT than of PPD.

By adhering to the official recommendation to revaccinate negative reactors to the second-strength dose of PPD (who reacted strongly with the second-strength dose of OT), the writers observed almost regularly an accelerated and

intensely inflamed local reaction in the vaccination punctures within two to six days after vaccination in contrast to the slowly developing vaccination papules in three to four weeks in negative reactors to the second-strength dose of both OT and PPD (7). This well-known accelerated phenomenon is widely accepted to indicate specific sensitization with BCG in spite of the negative skin reaction to PPD. The experience related was confirmed throughout the New York State BCG clinics. After having received numerous complaints about the apparent inadequacy of PPD as post-vaccination test material for tuberculin sensitivity in successfully vaccinated subjects, the present writers were constrained to modify the rules for tuberculin testing with preference to 0.1 and 1.0 "mg." of OT.

This experience revealed a need for an improved purified tuberculin for post-vaccination testing. The results of Seibert and her colleagues (8) showed that the A and B proteins were more active than the whole mixture of proteins contained in PPD; whereas the C protein, precipitable at pH 4.0, was less potent and possibly nonspecific. It appeared logical, therefore, to attempt the preparation of an improved tuberculin by methods that would yield the A and B fractions essentially free from C as well as from inert components. After several trials along this line, one partly purified protein (48189 #1, Nos. 14 and 15), which will be referred to as PT (prepared by M. C. P. and E. C.), proved sufficiently interesting to be given an extended experimental and clinical trial.

The following comparative study of PT, OT, and PPD was made possible by the kind cooperation of Dr. Howard C. Stewart, of the Division of Tuberculosis Control; Dr. Harry C. Storrs, Director of the Letchworth Mental Hygiene Institution; Dr. Reuben J. Erickson, Director of the Tuberculosis Division in the Albany Hospital, and his assistant, Miss Florence Nellis, R.N.; Dr. John C. Vanatta, Student Health Physician at the Southwestern Medical School of the University of Texas; Dr. Martin M. Cummings, Chief of the Tuberculosis Research Laboratory in the Veterans Administration Hospital, Chamblee, Georgia; and numerous volunteers in the Division of Laboratories and Research.

## PLAN OF INVESTIGATION

### *Clinical Material*

The clinical material consisted of three groups of persons with recent chest roentgenograms which showed no evidence of disease of the lungs. The control group contained 1,072 persons in a mental hygiene institution, hospital personnel, and laboratory workers with no history of tuberculosis.

The age distribution of the group was 10.5 per cent between 5 and 10 years, 53.8 per cent between 11 and 20 years, 21.3 per cent between 21 and 30 years, 10.1 per cent between 31 and 50 years, and 4.1 per cent up to 79 years of age. The BCG group consisted of 870 persons who had been BCG vaccinated by the multiple puncture method mostly within the previous twelve months, half of whom were inmates in a mental hygiene institution; and the remainder, medical students, student and graduate nurses, and laboratory personnel. Their age distribution was 18.6 per cent between 5 to 15 years and 81.4 per cent up to 30 years of age. The tuberculin-positive group consisted of 182 persons in a mental hygiene institution and laboratory personnel who during the previous year had been found positive to either the first- or second-strength doses of OT, but with no history of clinical tubercu-

losis. This group ranged in ages from 10 to 32 years. In a separate paper, Drs. H. M. DuBose, W. R. Mason, and M. M. Cummings will report on the comparative specificity and potency of PT and PPD in a series of known tuberculosis patients, BCG-vaccinated subjects, and healthy controls (16).

### *Tuberculins and Testing Methods*

Old Tuberculin, Lot 52, expiration date September 14, 1952, was prepared and standardized by the Division of Laboratories and Research (9). First- and second-strength doses were made in phosphate buffer solution pH 7.3 in 1:10,000 and 1:100 dilutions, containing per 0.1 ml., respectively, 0.01 and 1.0 "mg." of OT. Fresh dilutions were made before use and were always discarded within forty-eight hours after preparation. For comparison, a single lot of commercial purified protein derivative (Parke, Davis & Co., Lot Nos. 015889-X and 018490-Y, expiration date September 22, 1953) was used. First- and second-strength dose tablets were dissolved in the provided buffer solution to contain per 0.1 ml., respectively, 0.00002 and 0.005 mg. PPD. It should be noted that, although 0.005 mg. of PPD is 250 times stronger than 0.00002 mg. of PPD, its level of tuberculin activity is equivalent to 1.0 "mg." of OT (1). Fresh dilutions were made before use and discarded the same day.

The *Purified Tuberculin PT* was prepared as follows:

Cultures of the human strain of *M. tuberculosis* No. 48189 were grown on Long's medium for eight weeks, then killed by heating at 100°C. for one hour. The filtrates were dialyzed and dried from the frozen state. The fraction precipitable at pH 4.0 was removed and the supernatant adjusted to pH 3.5, ionic strength 0.1, and final concentration of 20 per cent alcohol by appropriate additions of acetate buffer and alcohol. The precipitate which separated under these conditions constituted the *Purified Tuberculin PT*. Temperatures were maintained at 0°C. or lower as long as alcohol was present.

Fraction PT represented approximately 20 per cent of the total nitrogen of the crude culture filtrate. It contained 6 per cent polysaccharide and gave no color test for desoxyribonucleic acid with diphenylamine. Electrophoresis revealed two components with suggestion of a third. The two peaks were not well resolved; the mobilities were approximately $4.88 \times 10^{-5}$ and $3.08 \times 10^{-5}$ at pH 7.7. Thus, the properties of the heat-denatured proteins present in the PT cannot be directly correlated with the values reported for native A and B proteins (8). Nitrogen was determined by the Kjeldahl method and the concentration of protein was estimated from the nitrogen content. Dosage was based on this analysis, so that the protein content of the test solutions was strictly comparable to that of the PPD solutions. First- and second-strength doses were prepared by diluting the sterile concentrated material in phosphate buffer solution pH 7.3 to contain per 0.1 ml., respectively, 0.00002 mg. and 0.005 mg. of PT. Fresh dilutions were made before use and discarded the same day.

New 1 cc. tuberculin syringes and 26-gauge intradermal point needles were used and kept separate for each type and dilution of tuberculin throughout the study. After each testing session, syringes and needles were thoroughly cleaned in 45 per cent urea during twenty-four hours, followed overnight by rinsing in running water when they were dried and autoclaved. The midvolar surface of the left forearm was used for injection sites for the first-strength doses and, after failing to react forty-eight hours later, the same surface of the right forearm was used for injection sites for the second-strength doses.

Reading of the tuberculin reactions was made regularly after forty-eight hours and never later than after seventy-two hours. The minimum requirement for a positive reaction was definite induration or edema 6 by 6 mm. in diameter and 3 mm. thick when skin was folded between caliper prongs. One-half of this thickness multiplied by the two maximum diameters of indurated area (for example: 1.5 by 6 by 6 mm. = 54 $mm.^3$) provided an estimate of the volume of induration. No posi-

tive reaction was recorded on the basis of erythema alone. The minimum of 6 by 6 mm. induration, irrespective of erythema, is the criterion for reading a positive test as recommended by the official BCG Vaccination Committee of New York (4). It has been found that the measurement of thickness of induration affords a more accurate means for reading doubtful positive reactions than the sole reading of surface measurements of induration.

## RESULTS

Rather than arbitrarily classifying positive reactions as one, two, three, or four plus, according to the extent of maximum induration diameters, all of the readings were measured as accurately as possible with millimeter calipers as in previous studies from this laboratory (10). Such volumetric measurements facilitate statistical analysis of possible differences in degree of induration produced with comparable strength dosages of the three tuberculins under discussion.

TABLE 1

COMPARISON OF POSITIVE TUBERCULIN REACTIONS FORTY-EIGHT HOURS AFTER INTRACUTANEOUS INOCULATIONS OF FIRST- AND SECOND-STRENGTH DOSES OF 0.01 AND 1.0 MG. OF OT AND 0.00002 AND 0.005 MG. OF PPD AND PT

| GROUP | TYPE OF TUBERCULIN | FIRST-STRENGTH DOSE | | | SECOND-STRENGTH DOSE | | | TOTAL | |
|---|---|---|---|---|---|---|---|---|---|
| | | Number Tested | Number Positive | Per Cent Positive | Number Tested | Number Positive | Per Cent Positive | Number Positive | Per Cent Positive |
| Control | OT | 150 | 81* | 54.0 | 69 | 45 | 65.2 | 126 | 84.0 |
| | PPD | 1,072 | 328 | 30.5 | 754 | 427 | 56.6 | 745 | 70.4 |
| | PT | 1,072 | 464* | 43.2 | 608 | 376 | 61.8 | 840 | 78.3 |
| BCG | OT | 127 | 64* | 50.4 | 63 | 62* | 98.4 | 126 | 99.0* |
| | PPD | 427 | 74 | 17.3 | 517 | 405 | 78.3 | 479 | 81.0 |
| | PT | 427 | 233* | 54.5 | 358 | 345* | 96.3 | 578 | 97.8* |
| OT-positive reactors, 1948 | PPD | 182 | 145 | 79.6 | 37 | 29 | 78.3 | 174 | 95.6 |
| | PT | 182 | 155 | 85.1 | 27 | 21 | 77.7 | 176 | 96.7 |

* The difference between these figures and those for PPD is significant by

$$\chi^2 \frac{(ad - bc)^2(a + b + c + d)}{(a + b)(c + d)(a + c)(b + d)}$$

where $a$, $b$, $c$, and $d$ are the four observed numbers.

In table 1 may be seen comparison of the positive reactions obtained with first- and second-strength doses of tuberculin in terms of percentages. It will be noted in the control group that the first-strength dose of OT and PT elicited significantly more positive reactors than PPD, but by retesting negative reactors with second-strength doses the number of positive reactors with PPD was not significantly less than with either OT or PT. The total percentages of positive reactors were approximately equal for the three tuberculins.

In the group of known tuberculin-positive reactors, there was a close correlation of reactors to the first- and second-strength doses of PPD and PT.

The most interesting discrepancy in response to the various tuberculins was

observed in the group of BCG-vaccinated subjects. Whereas a close equivalency obtained between positive reactors to first- and second-strength doses of PT and OT, the difference between the percentages of persons reacting positively with both strengths of PPD and OT or PT was significant. This is particularly marked with the first-strength dose of PPD, which elicited only 17.3 per cent positive reactors against more than 50 per cent with either OT or PT. With the second-strength dose, approximately 20 per cent less positive reactors were detected with PPD than with the two other tuberculins; and the total percentage of positive reactors with PPD still lagged approximately 20 per cent behind those detected by either OT or PT.

TABLE 2

COMPARISON OF VOLUME OF INDURATION (MM.$^3$) OF POSITIVE TUBERCULIN REACTIONS ($\geq 6 \times 6 \times 1.5$ MM. = 54 MM.$^3$) FORTY-EIGHT HOURS AFTER INTRACUTANEOUS INOCULATIONS OF FIRST- AND SECOND-STRENGTH DOSES OF OT, PPD, AND PT

| GROUP | TYPE OF TUBERCULIN | FIRST-STRENGTH DOSE | | SECOND-STRENGTH DOSE | |
|---|---|---|---|---|---|
| | | Number Positive | Induration (Mm.$^3$) | Number Positive | Induration (Mm.$^3$) |
| Control | OT | 81 | 610 ± 770* | 45 | 1,476 ± 1,404* |
| | PPD | 328 | 443 ± 463 | 427 | 482 ± 762 |
| | PT | 464 | 2,560 ± 2,115* | 376 | 1,017 ± 1,316* |
| BCG | OT | 64 | 160 ± 112 | 62 | 1,127 ± 853* |
| | PPD | 74 | 163 ± 117 | 405 | 690 ± 668 |
| | PT | 233 | 399 ± 266* | 345 | 2,409 ± 1,919* |
| OT-positive reactors, 1948 | PPD | 145 | 533 ± 333 | 29 | 1,055 ± 787 |
| | PT | 155 | 3,700 ± 2,759* | 21 | 1,614 ± 1,068 |

* The difference between these figures and those for PPD is significant by

$$t = \frac{x - x'}{s} \sqrt{\frac{(n_1 + 1)(n_2 + 1)}{n_1 + n_2 + 2}}$$

at the $P = 0.01$ level.

Standard deviation ($\pm$): $\sigma = \sqrt{\frac{S(d^2)}{n - 1}}$

Differences in the volume of induration produced by first- and second-strength doses of the three tuberculins are presented in table 2. As may be seen, the first-strength dose of PT produced significantly greater volumes of induration than either OT or PPD in the three groups under investigation. The least volume of induration was produced by PPD, except in BCG-vaccinated subjects who responded equally well to the first-strength dose of OT and PPD. As might have been expected, a considerably greater area of induration to the first-strength dose of the various tuberculins was observed in naturally infected subjects than in BCG-vaccinated subjects. Nevertheless, the personnel who participated in these field studies were unanimous in their opinion that the reading of the first-strength dose reactions with OT and PT was considerably easier than with PPD,

which usually produced a poor to fair induration reaction of 1 and 2 plus intensity in contrast to the markedly raised and definitely circumscribed induration reactions of 2 and 3 plus intensity produced by OT and especially by PT.

In view of the excessively intense areas of induration produced by the second-strength dose of OT and PT in successfully BCG-vaccinated subjects who failed to react positively to the first-strength dose, as well as the higher frequency of pseudoreactions with the second-strength dose, comparative tuberculin tests were made with an intermediate strength of 0.1 "mg." of OT and 0.0002 mg. of PT and PPD. The comparative tests were made in a series of 66 student nurses successfully vaccinated with BCG by the multiple puncture method eight to sixteen weeks previously. The data on positive reactors and volumes of induration are presented in table 3. It will be noted that 96.9 per cent reacted equally positively with OT and PT, whereas only 56.1 per cent reacted positively with PPD. The occurrence of pseudoreactions was almost negligible with this intermediate-strength dose of OT and PT and distinctly better induration reactions to either

TABLE 3

COMPARISON OF POSITIVE REACTORS AND VOLUME OF INDURATION (MM.²) WITH INTERMEDIATE STRENGTH DOSE OF 0.1 MG. OF OT AND 0.0002 MG. OF PPD AND PT IN BCG-VACCINATED SUBJECTS

| TYPE OF TUBERCULIN | INTERMEDIATE STRENGTH DOSE OF TUBERCULIN | | | |
|---|---|---|---|---|
| | Number Tested | Number Positive | Percentage Positive | Volume of Induration (Mm.²) |
| OT | 66 | 64* | 96.9 | 301 ± 185* |
| PPD | 66 | 37 | 56.1 | 185 ± 114 |
| PT | 66 | 64* | 96.9 | 461 ± 262* |

* Significant differences from PPD figures.

OT or PT were observed than to PPD. Thus, it would appear that a single pre-vaccination and post-vaccination test with 0.1 "mg." of OT or 0.0002 mg. of PT is adequate to detect the appearance of tuberculin sensitivity in successfully vaccinated subjects without inflicting excessively severe local reactions.

## DISCUSSION

It is generally agreed that the ideal tuberculin antigen and tuberculin test have not as yet been devised (11). Such an antigen would contain the whole protein molecule necessary for complete tuberculin action (12), and the tuberculin test would have a sharp endpoint with an accurately measurable reaction in terms of induration. Substitution of commercial purified protein derivative (PPD) for Old Tuberculin seemed warranted after the demonstration that: the skin-reacting specificity and potency of tuberculin depends solely on its protein content and not on such impurities as nucleic acid and polysaccharide (1); and the specific tuberculin reaction of PPD was almost equivalent to OT in patients with bacteriologic and roentgenographic evidence of pulmonary tuberculosis (2). A deficiency of exclusive use of PPD as test material was brought out in

the present investigation of the comparative specificity and potency of OT, the purified tuberculin (PT), and PPD in persons successfully vaccinated with BCG. In this group approximately 30 per cent fewer positive reactors were observed with the first-strength dose and approximately 20 per cent less with the second-strength dose of PPD than with corresponding doses of either OT or PT. On the supposition that PPD is equivalent in specificity and potency to OT in all persons infected with tubercle bacilli (including BCG organisms), all negative reactors to PPD among apparently successfully BCG-vaccinated subjects are advised to be revaccinated if tuberculin sensitivity has not appeared eight to twelve weeks after vaccination. This practice has invariably led to an untoward production in such persons of an accelerated vaccination reaction (Koch phenomenon) within a few days after revaccination (7) and subsequent blemishing pigmentation and scarification marks. On the basis of such observations, Ustvedt (13) has questioned the reliability of the tuberculin test among negative reactors in successfully vaccinated subjects and he recommends instead the use of the Koch phenomenon, elicited by applying viable or heat-killed BCG vaccine by the multiple puncture method (2 punctures), as a substitute for the tuberculin test. This view is also supported by Aronson and McGettigan (14).

The present study has also brought out that the sole use of the first-strength dose of PPD in general diagnostic and epidemiologic work fails to detect approximately 25 per cent of subjects who react positively with corresponding doses of either OT or PT. This significant difference in specificity between PPD and the two other tuberculins with the first-strength dose is important in view of the observation by Furcolow and his co-workers (15) that the use of the first-strength dose is less productive of "false" or "unspecific" reactions than is the second-strength dose, which is capable of producing some sort of intracutaneous reaction in all tested subjects. Nevertheless, in previously known tuberculin-positive reactors to OT, the present writers observed complete equivalence between PPD and PT, and the same will be reported in a separate paper by DuBose, Mason, and Cummings (16) in patients with active tuberculosis.

It should be emphasized that the tuberculin fraction PT is not a "pure" protein, but is merely the most potent fraction that it was possible to isolate by simple methods from heated culture filtrates. More satisfactory purification can undoubtedly be attained by starting with unheated filtrates. It is believed that the importance of the present results lies in the demonstration that such a partly purified fraction may be a more reliable and specific indicator to tuberculin sensitivity in BCG-vaccinated persons than is the whole mixture of active proteins. This lends weight to the argument that efforts at preparing a true "purified" standard should be directed to the use of individual proteins such as Seibert's A and B fractions (8), rather than to recovering the entire tuberculin activity of the filtrates.

## SUMMARY

A comparative study has been made of the specificity and potency of Old Tuberculin (OT), purified protein derivative (PPD), and a purified tuberculin (PT) in healthy control subjects, known tuberculin-positive reactors to OT, and BCG-vaccinated persons.

The percentage of positive reactions induced in all groups by the first- and second-strength doses (0.00002 and 0.005 mg.) of the PT used in this study was equivalent to that induced by 0.01 and 1.0 "mg." of OT, while PPD produced significantly less positive reactions to the first-strength dose in healthy controls, and to first- and second-strength doses in BCG-vaccinated persons. Equivalence between PT and PPD was observed in known OT-positive reactors.

The purified tuberculin used in this study would appear more reliable than purified protein derivative to detect the appearance of tuberculin sensitivity in BCG-vaccinated persons whose acquired tuberculin sensitivity is significantly less than that produced by a natural infection with tubercle bacilli.


## SUMARIO

*Estudios de una Fracción Purificada de Tuberculina en la Comprobación de los Sujetos Vacunados con BCG. Estudio Preliminar*

Este estudio comparado de la especificidad y la potencia de la Tuberculina Antigua (TA), del derivado proteínico purificado (DPP), y de una tuberculina purificada (TP) fué llevado a cabo en testigos sanos, reactores conocidos a la tuberculina (TA) y personas vacunadas con BCG.

El porcentaje de reacciones positivas evocadas en todos los grupos con dosis de la primera y la segunda dilución (0.00002 y 0.005 mg.) de la TP usada en este estudio fué equivalente al evocado con 0.01 y 1.0 "mg." de TA, mientras que el DPP produjo reacciones significativamente menos positivas con dosis de la primera dilución en testigos sanos, y con dosis de la primera y la segunda dilución en los becegetizados. En los reactores conocidos a la TA hubo equivalencia entre la TP y el DPP.

La tuberculina purificada utilizada en este estudio parece ser más digna de confianza que el derivado proteínico purificado para descubrir el viraje a la tuberculina en los becegetizados cuya sensibilidad adquirida a la tuberculina es significativamente menor que la producida por una infección natural con bacilos tuberculosos.

## RESUME

*Etude d'une fraction de tuberculine purifiée au cours de tests chez des sujets vaccinés par le BCG. Etude préliminaire*

Une étude comparative sur la spécificité et l'activité de la Vieille tuberculine (OT), du dérivé protéique purifié (PPD) et d'une tuberculine purifiée (PT) a été effectuée chez des sujets témoins en bonne santé, des sujets ayant une réaction positive à la tuberculine (OT) et chez des sujets vaccinés par le BCG.

Le pourcentage de réactions positives, provoqué dans tous les groupes par la dose No. 1 et la dose No. II (0,00002 et 0,005 mg.) de PT utilisé dans cette étude, s'est montré équivalent à celui provoqué par 0,01 et 1 "mg." de OT, tandis que le nombre de réactions positives produites par le PPD à la dose No. 1 chez les témoins bien portants et aux doses No. 1 et No. II chez les sujets vaccinés par le BCG étaient nettement moins significatif. L'équivalence entre PT et PPD a été observée chez les sujets ayant une réaction positive à OT.

Il semble que la tuberculine utilisée dans cette étude soit plus sûre que le dérivé protéique purifié, pour déceler la conversion tuberculinique chez les individus vaccinés par le BCG, dont la sensibilité acquise à la tuberculine est nettement inférieure à celle résultant de l'infection naturelle par le bacille tuberculeux.


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Personal communication.

# COMPARISON OF A NEW PURIFIED TUBERCULIN WITH PURIFIED PROTEIN DERIVATIVE[1,2]

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## INTRODUCTION

The discovery of Old Tuberculin (OT) by Robert Koch (1) in 1890 aided immeasurably in the study of the epidemiology of tuberculosis. With the preparation of OT in many laboratories throughout the world, however, wide variations in the skin reactions produced by these preparations became apparent, seriously impairing its use as an epidemiologic and a diagnostic tool. This was pointed out by Calmette and de Potter (2) in 1926 in a survey showing the variations in methods of preparation and potency of OT produced from different strains of the tubercle bacillus under identical conditions in the same laboratory. It was also recognized that the undesirable sensitizing property of OT which produced shock in the guinea pig was caused by a different principle from that responsible for the skin reaction.

In an attempt to standardize and purify tuberculin, Long and Seibert (3) isolated a protein with high tuberculin activity from a synthetic culture medium in which tubercle bacilli had been grown. This substance proved to be the active principle of the tuberculoprotein that produced the skin reaction in sensitive individuals only and yet did not produce skin sensitivity in nonallergic subjects. It was introduced in 1934 as purified protein derivative (PPD) (4) followed in 1938 by another practical purified tuberculin by Jensen and his co-workers (5). The great advantage of this substance was its availability in dry form without loss of potency under long storage conditions, and the increased accuracy with which it could be prepared for clinical use as compared with the uncertain dilution factors of the OT stock solutions. The use of PPD in large series has shown its dependability as a uniform skin-testing material, permitting the comparison of the results obtained in different groups. This material, in the standard first- and second-strength doses of 0.00002 mg. and 0.005 mg., respectively, was found to elicit skin reactions in 90 to 95 per cent of persons with known tuberculous infections (4, 6).

An ideal tuberculin skin-testing substance might be defined as a chemically stable substance which, in a single measured quantity, will produce, without systemic or severe local side effects, a skin reaction in all individuals sensitive to tuberculoprotein by natural or artificial infections, but will not produce skin reaction or induce tuberculoprotein sensitivity in nonsensitive individuals. In

[1] From the Tuberculosis Research Laboratory, Veterans Administration Hospital, Chamblee, Georgia; and the Department of Medicine, Emory University, School of Medicine, Atlanta, Georgia.

[2] Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own studies and do not necessarily reflect the opinion or policy of the Veterans Administration.

spite of the tremendous advances made in the last thirty years, this goal has not been reached. With the advent of mass BCG vaccination there has been a surge of interest in the application of the tuberculin test. Of particular importance is the observed discrepancy between the number of "takes" after intradermal injection of the vaccine and the considerably lower percentage of tuberculin reactions six to twelve weeks afterwards.

Because of the important role of the tuberculin test in BCG vaccination, it seems desirable to improve its reliability whenever possible. As a consequence, the present study was made in which the new tuberculin (Pangborn-Cummerow-Birkhaug) skin-testing substance has been compared with PPD (14). The properties of the Pangborn-Cummerow-Birkhaug material are presented in another report in this journal (page 335).

## MATERIALS AND METHODS

### *Subjects*

Three groups were chosen for the study. The first was a control group consisting of general medical patients and hospital personnel without known tuberculosis; the second was composed of known tuberculous patients; the third group consisted of medical students, nurses, and hospital attendants, who had been vaccinated with BCG within the preceding eight months. All subjects had recent 14- by 17-inch chest roentgenograms.

### *Tuberculins*

The new tuberculin was identified as Purified Tuberculin #48189, Lot 14, 15, and was prepared in the Division of Laboratories and Research, New York State Department of Health[3]. For convenience, it will be designated hereafter as *PT*. For comparison, a single lot of commercial purified protein derivative (Parke, Davis & Company) was used and will be designated PPD. The PT was made available in stock solutions and was prepared in first strength (0.00002 mg.) and second strength (0.005 mg.) doses as needed. PPD was available in tablet form and was prepared in first-strength (0.00002 mg.) and second-strength (0.005 mg.) doses as needed, using the buffered diluent provided. Both solutions were refrigerated when not in use and were discarded when seventy-two hours old.

New, unused, 1 cc. tuberculin syringes and 26-gauge needles were used. Each tuberculin had its assigned syringes and needles, which were kept separate during the entire study. Syringes and needles were washed thoroughly with warm soapy water, rinsed several times in clear tap water, and autoclaved when not in use or when changing from first- to second-strength solutions (7). The midvolar surfaces of the forearms were used for injection sites and, if no reaction occurred within forty-eight hours after the first-strength dose, second-strength doses were injected at sites level with or distal to the previous injection sites.

Reactions were examined forty-eight hours after injection by the same observer who made the injections. Three observers performed the tests. One tested 168 subjects; a second, 25 subjects; and a third observer, 22. A reaction was considered positive when 5 mm. or more induration of the skin with erythema was found by actual measurement at the site of injection. Erythema itself was not

[3] Supplied to us by Dr. Konrad Birkhaug, Dr. Mary C. Pangborn, and Mrs. Elizabeth Cummerow of the Division of Laboratories and Research, New York State Department of Health, Albany, New York.

considered to be indicative of positive reaction and, therefore, was estimated but not recorded in this report.

TABLE 1

COMPARISON OF POSITIVE SKIN REACTIONS AT THE END OF FORTY-EIGHT HOURS AFTER INTRACUTANEOUS INJECTIONS OF PURIFIED PROTEIN DERIVATIVE (PPD) AND PURIFIED TUBERCULIN (PT)

| GROUP | TYPE OF TUBERCULIN | 0.00002 MG. FIRST STRENGTH | | | 0.005 MG. SECOND STRENGTH | | |
|---|---|---|---|---|---|---|---|
| | | Number Tested | Number Positive | Per Cent Positive of Total | Number Tested | Number Positive | Per Cent Positive of Total |
| Control | PPD | 99 | 38 | 38.0 | 29 | 17 | 60.0 |
| | PT | 99 | 53 | 53.0 | 29 | 20 | 70.0 |
| Tuberculosis | PPD | 51 | 46 | 90.0 | 2 | 1 | — |
| | PT | 51 | 49 | 96.0 | 2 | 2 | — |
| BCG | PPD | 65 | 10 | 15.4* | 30 | 22 | 73.0 |
| | PT | 65 | 32 | 50.0* | 30 | 25 | 83.0 |

* The difference between these figures is significant by $T = \frac{P_1 - P_2}{\alpha P_1 - \alpha P_2}$.

TABLE 2

COMPARISON OF INDURATION OBTAINED AT THE END OF FORTY-EIGHT HOURS AFTER INTRACUTANEOUS INOCULATION WITH PURIFIED PROTEIN DERIVATIVE (PPD) AND PURIFIED TUBERCULIN (PT)

| GROUP | TYPE OF TUBERCULIN | 0.00002 MG. FIRST STRENGTH | | 0.005 MG. SECOND STRENGTH | |
|---|---|---|---|---|---|
| | | Number Positive | Average Induration (Millimeters) | Number Positive | Average Induration (Millimeters) |
| Control | PPD | 38 | 9.0 | 17 | 9.0 |
| | PT | 52 | 13.5 | 20 | 12.0 |
| Tuberculosis | PPD | 46 | 10.5 | 1 | — |
| | PT | 49 | 15.0 | 2 | — |
| BCG | PPD | 10 | 7.3 | 22 | 13.0 |
| | PT | 32 | 13.2 | 25 | 16.5 |

Differences in each group were compared by $T = \frac{P_1 - P_2}{\alpha P_1 - \alpha P_2}$.

## RESULTS

*Positive reactors:* There were more positive reactors to PT than to PPD (table 1). In the control group 53 (53 per cent) of those receiving the first-strength dose gave positive reactions to PT as compared with 38 (38 per cent) for PPD. Of those in the control group who were given the second-strength dose, 20 (70 per

cent) reacted to PT and 17 (60 per cent) to PPD. Nevertheless, these differences are not significant. Forty-nine (96 per cent) of the tuberculous group had positive skin reactions to first-strength PT while 46 (90 per cent) reacted to first-strength PPD. It is interesting to note that, in the BCG group, 32 (50 per cent) reacted to first-strength PT, but only 10 subjects (15.4 per cent) reacted to a similar dose of PPD. Thirty subjects in this group were given the second-strength dose of the two tuberculins, with 25 (83 per cent) reacting to PT and 22 (73 per cent) reacting to PPD. This was not a significant difference for the second-strength tuberculins, but a statistically significant difference exists when the first-strength results are compared.

*Induration:* It was thought that a comparison of the induration produced by the two tuberculins would be of interest. These values are set forth in table 2. It will be noted that the average induration in millimeters was greater for PT in all groups except the tuberculous patients who were given second-strength doses of the two tuberculins. In each case the difference is statistically significant.

## DISCUSSION

A comparison of the potency of two tuberculin preparations is at best a crude estimation because such a study is fraught with many unavoidable errors in technique as well as the inevitable biologic variations. Variations may occur in preparing small quantities of biologicals for clinical use. Moreover, it is evident that variations must occur in the actual injection of tuberculin. When it comes to individual interpretation of skin reactions, one has only to refer to the recent study by Meyer, Hougen, and Edwards (8) to appreciate the wide range of individual variation. In the present study it was sought to reduce the "human error" by having each observer prepare all solutions, perform all injections, and interpret all skin reactions for one specific group of subjects. Undoubtedly, small quantities of tuberculin remain on the syringe and needle after use, as was shown by Nelson, Seibert, and Long (7), unless special attention is given to the cleansing of these instruments. This could be the source of considerable error if the same syringe were used interchangeably for two strengths of tuberculin. It was believed that cleaning the equipment as described was adequate to reduce this source of error to a minimum. The question of variability in potency of different lots of tuberculins does not enter into this study, as single lots of each type were used.

Unfortunately, all persons who have tuberculous infections will not give a positive skin reaction to tuberculin, regardless of the potency of the tuberculin used (9, 10, 11). For the most part, these are cachetic or senile patients with active tuberculosis who lose their tissue allergy and usually do not present a great problem. There were no such patients included in the present study.

It is well known that BCG-vaccinated subjects frequently lose their positive tuberculin reaction within various periods of time following vaccination and that some never react to tuberculin (9, 12). The BCG group in the present study had been vaccinated by the multiple puncture technique six to eight months prior to the study with the same lot of vaccine, and all reacted to tuberculin six weeks later. It is interesting to note that six to eight months later only 15.4 per cent

reacted to PPD but 50.0 per cent reacted to PT, and the average size of skin reaction to PT was significantly greater than the reaction to PPD. If PT is truly a more sensitive indicator of tuberculin sensitivity than PPD, it would be of great benefit in more accurately determining the incidence or "takes" and persistence of allergy among BCG-vaccinated subjects.

In contrast to infected individuals with negative tuberculin skin reactions, there are those who have a positive skin reaction to tuberculin but are not infected and apparently have never had known contact with the tubercle bacillus. This has been referred to as the "false positive" or nonspecific tuberculin reaction. Furcolow and his associates (13) in 1941, employing quantitative tuberculin skin tests, found that all persons will react to tuberculin if the quantity given is large enough, regardless of absence of tuberculosis clinically or history of exposure. In Furcolow's tuberculous group, 99 per cent reacted to first- and second-strength doses ordinarily used clinically. This was experienced in the tuberculous group of the present study with both PPD and PT. Ustvedt and Aanonsen believe that "false positives" or nonspecific reactions to tuberculin occur more frequently when the intracutaneous method of testing is used (11).

Despite the fact that 98 per cent of the tuberculous group reacted to PPD and 100 per cent to PT, the possibility of "false positive" or nonspecific reactions cannot be excluded and the problem must be studied in more detail in a large series. These writers (11) have suggested the use of the diagnostic BCG test as a means to distinguish between the nonspecific and true reactions to tuberculin. The greater number of positive reactions and the consistently larger skin reactions obtained from PT in the first strength as compared with PPD in like quantity seems to indicate that PT is a more sensitive indicator of tuberculin skin sensitivity than PPD.


## SUMMARY

A new purified tuberculin and commercial purified protein derivative were compared in apparently normal, tuberculous, and BCG-vaccinated persons.

The purified tuberculin yielded a greater number of positive reactors in the first-strength dose than did purified protein derivative, but the difference is significant only in the BCG group. The induration was more intense and larger when the new purified tuberculin was used. This was more striking in the group of persons tested six to eight months after BCG vaccination. There was no significant difference in the number of reactions obtained by the use of the second-strength doses of the two tuberculins, but the size of reaction produced by the purified tuberculin (Pangborn-Cummerow-Birkhaug) was significantly greater.

## SUMARIO

*Comparación de una Nueva Tuberculina Purificada con DPP*

La nueva tuberculina purificada y el derivado proteínico purificado comercial (DPP) fueron comparados en personas aparentemente sanas, tuberculosas y vacunadas con BCG.

La tuberculina purificada dió un número mayor de reactores positivos con dosis de la primera dilución que el derivado proteínico purificado, pero la diferencia sólo revistió importancia en el grupo becegetizado. La induración fué más intensa y mayor cuando se usó la nueva tuberculina purificada, siendo esto más notable en el grupo de personas compro-

badas de seis a ocho meses después de la vacunación con BCG. No hubo diferencia significativa en el número de reacciones obtenidas al usar dosis de la segunda dilución de las dos tuberculinas, pero fué significativamente mayor el tamaño de la reacción producida por la tuberculina purificada (Pangborn-Cummerow-Birkhaug).

RESUME

*Etude comparative d'une tuberculine purifiée nouvelle et du PPD*

Une étude comparative de la nouvelle tuberculine purifiée et du dérivé protéique purifié du commerce a été effectuée chez des sujets paraissant bien portants, chez des tuberculeux et chez des individus vaccinés par le BCG.

La tuberculine purifiée à la dose No. I (0,00002 mg.) a donné un nombre plus grand de sujets a réaction positive que le dérivé de protéine purifiée, mais la différence n'est significative que dans le groupe BCG. L'induration était plus intense et plus étendue quand la nouvelle tuberculine purifiée était employée. Cette manifestation était encore plus frappante dans le groupe de sujets soumis au test dans un délai de six à huit mois après la vaccination par le BCG. Il n'y avait pas de différence significative dans le nombre de réactions obtenues par l'usage de la dose No. 2 (0,005 mg.) des deux tuberculines, toutefois, la dimension de la réaction produite par la tuberculine purifiée (Pangborn-Cummerow-Birkhaug) était nettement plus grande.

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# THE SIGNIFICANCE OF THE TUBERCULIN SKIN REACTION IN ANTITUBERCULOUS VACCINE ASSAY[1]

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## INTRODUCTION

The significance of post-vaccination tuberculin allergy in BCG immunization has not been agreed upon. On the one hand, there has been the supposition and claim that post-vaccination tuberculin sensitivity is a direct measure, or at least an indication, of immunity while, on the other hand, it has been suggested that it may indicate no more than multiplication or deposition of the organisms in the tissues. Hertzberg (1), Ustvedt (2), and others (3, 4) believe that a moderately high sensitivity to tuberculin is an indication of a higher degree of resistance to infection than is a slight sensitivity; whereas Aronson (5), Willis (6), Rich, and McCordock (7) and others (8, 9) suggest that there is not necessarily a relationship between the degree of immunity and allergy. The latter point of view suggests that acquired immunity may develop coincidentally with the allergenic response but that its coexistence may not be invariable. Raffel (8) has demonstrated that guinea pigs immunized with certain components alone of the tubercle bacillus may develop cutaneous allergic reactivity to tuberculin but show no resistance to tuberculous infection, thus emphasizing the possibility of the independence of tuberculin sensitivity and acquired immunity.

Inasmuch as the evaluation of an antituberculous vaccine by the immunization, challenge, and subsequent post-mortem examination of animals is a procedure of long duration, a more rapid indicative measure of immunity would be a useful additional tool. The post-vaccination tuberculin sensitivity would, therefore, be a comparatively simple expedient for the quantitative evaluation of immunity were it not for its controversial position. In a series of potency assays on a number of vaccines, an attempt has been made to assess the relation of the quantitative post-vaccination allergy to protective immunity as determined in guinea pig tests.

## MATERIALS AND METHODS

Two types of antituberculous vaccines, BCG and a nonviable irradiated vaccine prepared from a virulent culture of *M. tuberculosis* (10), were evaluated for their antigenic potency in guinea pigs. BCG vaccines were prepared in this laboratory and also obtained from two recognized BCG laboratories, and are designated hereafter as BCG *A*, *B*, and *C*. The BCG vaccines were always used within twenty-four to forty-eight hours of preparation. Groups of 30 to 35 tuberculin-negative, white, male, 325- to 375-gram guinea pigs were immunized by three weekly 5 mg. doses. BCG was given intraperitoneally while irradiated vaccines were given intraperitoneally, subcutaneously, intramuscularly, or intracutaneously.

Approximately one month following the last dose of vaccine and before challenge, 0.0005

[1] From the Research Laboratories, Parke, Davis & Company, Detroit, Michigan.

mg. (5/10,000) of tuberculin PPD was given intracutaneously, and the tuberculin reactions were recorded twenty-four hours later.

Following the determination of post-vaccination tuberculin sensitivity, all immunized animals and a control group of normal animals were challenged intraperitoneally with 0.01 mg. semi-moist weight of *M. tuberculosis* H37Rv.

Approximately two to three months after challenge, when random sampling showed progressive tuberculosis established in the controls, all animals were sacrificed and postmortem examinations were made to determine the extent of tuberculous involvement of the liver, spleen, and lungs. Potency of the vaccines was evaluated by a modification of the Feldman Index (11) in which arbitrary numerical values are assigned to the organs according to the degree of infection, considering 100 units as the maximal arbitrary theoretical

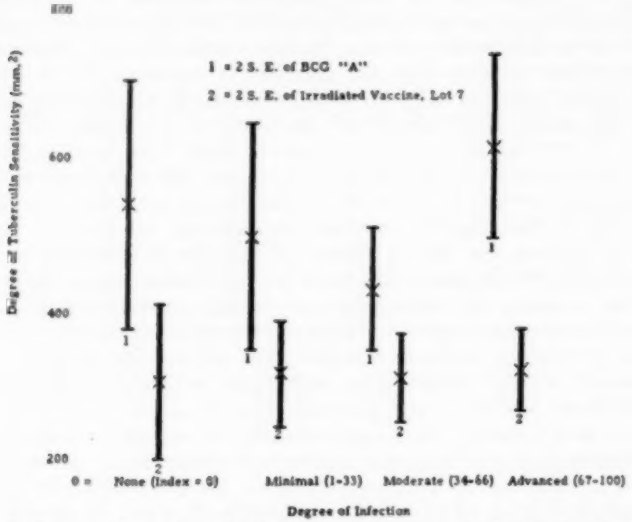


FIG. 1. Comparative post-vaccination allergy and degree of infection for BCG and irradiated vaccine.

amount of tuberculosis possible in any one animal. Animals were tabulated in groups on the numerical basis of the degree of immunity.

Within each of these immunity-based groups, the variants in the post-vaccination tuberculin sensitivity were plotted graphically as computed from the 2 Standard Error of the average area of the tuberculin reaction and the positive and negative deviations from that reaction.

## RESULTS

Results of comparative evaluations between BCG and irradiated vaccine are shown in figure 1. The 2 Standard Error "Spread" (or deviations) of the tuberculin reactions for the two vaccines are shown on the same graph, grouped according to the degree of tuberculous infection. The average tuberculin reaction of each group is shown as the intermediate point of the solid line while the devia-

tions from that point indicate the 2 Standard Error range of the tuberculin reactions. Inasmuch as a straight line can be drawn across the figure which will intersect the plottings of the tuberculin reactions of each immunity-based group of animals, there is no mathematical significance in the difference of degree of tuberculin sensitivity in those groups of animals immunized with the same vaccine but showing different degrees of immunity. In other words, in figure 1 the range of degree of tuberculin sensitivity in each group of animals, regardless of the quantitative extent of disease in the group, overlapped the range of tuberculin sensitivity in each of other groups in the BCG *A*-vaccinated animals which are

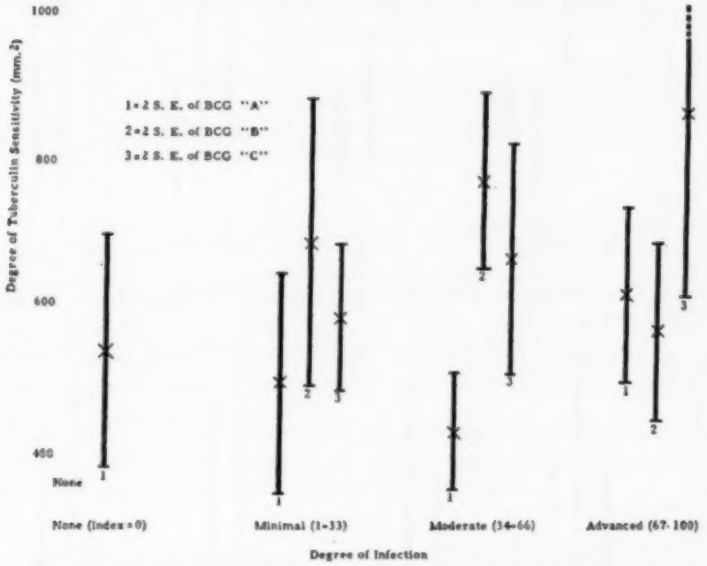


FIG. 2. Comparative post-vaccination allergy and degree of infection for three BCG vaccines.

designated by the numeral "1." The same held true for the animals immunized with irradiated vaccine and designated by the numeral "2."

Three BCG vaccines, *A*, *B*, and *C*, were also evaluated for their antigenic potency and post-vaccination tuberculin allergy. As in the preceding experiment, the 2 Standard Error tuberculin reactions of guinea pigs immunized with these vaccines have been plotted within each immunity-based group. In figure 2 it can be seen once again that between these immunity-based groups there was no significant correlation between the tuberculin reaction and the degree of immunity. None of the animals immunized with BCG *B* and BCG *C* fell in the "no tuberculosis infection" group, which accounts for the absence of these lines in the graph.

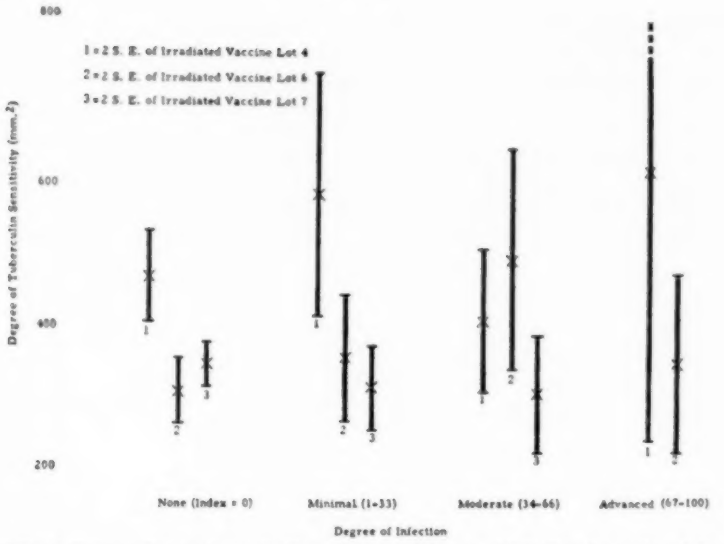
800
600
400
200
1 = 2 S. E. of Irradiated Vaccine Lot 4
2 = 2 S. E. of Irradiated Vaccine Lot 6
3 = 2 S. E. of Irradiated Vaccine Lot 7
Degree of Tuberculin Sensitivity (mm. 2)
None (Index = 0)
Minimal (1-33)
Moderate (34-66)
Advanced (67-100)
Degree of Infection

FIG. 3. Comparative post-vaccination allergy and degree of infection for various lots of rradiated vaccine.

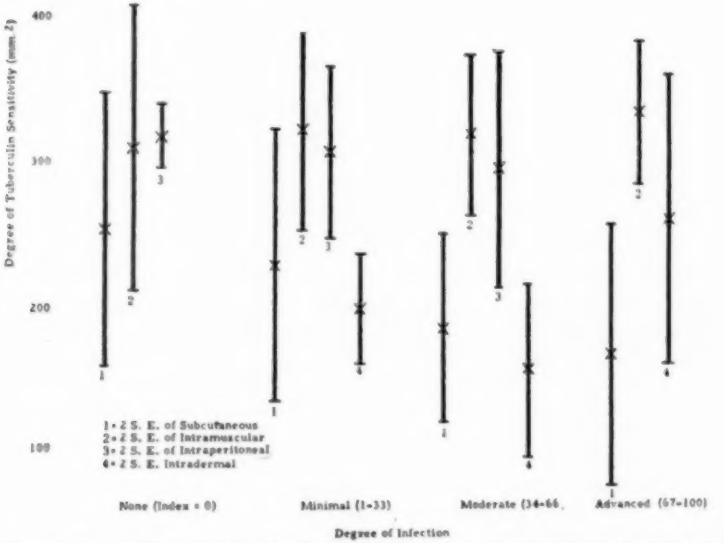


FIG. 4. Comparative post-vaccination allergy and degree of infection for irradiated vaccine by various routes of administration.

This same test was also applied to groups of guinea pigs immunized with three different lots of irradiated vaccine. In these experiments also (figure 3), the 2 Standard Error tuberculin reactions of these animals showed no correlation with the degree of immunity. It is interesting to note that, if only the average of the tuberculin reactions had been used in the group immunized with Lot 7 irradiated vaccine, there would erroneously appear to be a relation between the degree of tuberculin sensitivity and the amount of acquired immunity. Nevertheless, in these experiments also it can be seen that a straight line will bisect all the plottings, showing that here too, as well as with the other vaccines in this group, there was no significant correlation between the tuberculin sensitivity and the degree of immunity. None of the animals immunized with Lot 7 irradiated vaccine showed advanced tuberculosis.

The relation of post-vaccination allergy to protective immunity in animals immunized by various routes with the same irradiated vaccine is shown in figure 4. Again, in none of these groups was there a significant correlation between the tuberculin reactions and the degree of tuberculosis.


## SUMMARY

Guinea pigs immunized with either BCG or irradiated vaccines of dead bacilli given by different routes showed no significant correlation between the degree of post-vaccination tuberculin sensitivity before challenge and the degree of immunity following challenge with a virulent culture.

Plotting of the variants of the tuberculin reactions demonstrates that there is a wide degree of variation in the tuberculin sensitivity within, as well as between, groups of animals with a similar degree of immunity as well as in those animals with differing degrees of immunity.

On the basis of results obtained in these experiments, the post-vaccination tuberculin reaction is not a suitable quantitative measure of immunity for antituberculous vaccine assay under the test conditions used.

## SUMARIO

*La Significación de la Cutirreacción a la Tuberculina en la Valoración de Vacunas Antituberculosas*

Los cobayos inmunizados bien con BCG o con vacunas antituberculosas irradiadas administradas por distintas vías no revelaron correlación significativa entre la intensidad de la sensibilidad postvacunal a la tuberculina antes de la provocación y la intensidad de la inmunidad consecutiva a la provocación con un cultivo virulento.

El trazado de las variantes de las reacciones a la tuberculina demuestra que existe amplia variación de la sensibilidad a la tuberculina dentro de, así como entre, los grupos de animales dotados de inmunidad semejante, e igualmente en los animales dotados de distinta inmunidad.

A base de los resultados obtenidos en estos experimentos, la reacción postvacunal a la tuberculina no constituye una medida cuantitativa adecuada de la inmunidad para la valoración de vacunas antituberculosas en las condiciones de este ensayo.

## RESUME

*La valeur de la réaction cutanée à la tuberculine dans le titrage du vaccin tuberculeux*

Chez des cobayes ayant été immunisés soit avec le BCG soit avec des vaccins tuberculeux irradiés, administrés par des voies différentes, il n'a été observé aucune corrélation signifi-

cative entre les degrés de la sensibilité post vaccinale à la tuberculine avant le test et le degré de l'immunité obtenu à la suite d'un test avec une culture virulente.

Le graphique des variantes des réactions à la tuberculine prouve qu'il existe une grande gamme de variations dans la sensibilité à la tuberculine à l'intérieur des groupes d'animaux comme entre les groupes d'animaux possédant un degré d'immunité similaire, de même que chez ceux des animaux présentant des degrés d'immunité différents.

Sur la base des résultats obtenus dans ces recherches, la réaction post-vaccinale à la tuberculine n'est pas une mesure quantitative satisfaisante de l'immunité pour titrer le vaccin tuberculeux, dans les conditions de l'expérimentation.

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# HYPOPOTASSEMIA AND HYPONATREMIA IN PULMONARY TUBERCULOSIS DURING TREATMENT WITH STREPTOMYCIN AND PARA-AMINOSALICYLIC ACID[1,2]

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## INTRODUCTION

The present study was undertaken after noting hypopotassemia in 2 patients and hyponatremia in 3 others under treatment for tuberculosis with streptomycin and oral para-aminosalicylic acid (PAS). A potassium deficiency during PAS therapy has been reported (1, 2) although the mechanism causing this deficiency has not been clarified. An attempt was made in the present study to determine whether para-aminosalicylic acid therapy may be a causative factor in producing potassium and sodium deficiencies in tuberculosis.

## MATERIALS AND METHODS

Ten subjects with active pulmonary tuberculosis were studied. Therapy consisted of 1 gm. of streptomycin twice weekly and 12 gm. of PAS orally daily. The patients received a diet of 2,800 calories per day, containing an average of 3.6 gm. of sodium and 5.1 gm. of potassium with an added salt intake of 9 to 12 gm. of sodium (as NaCl) and 3.0 mg. of potassium. A rigid control of mineral intake was not feasible during this extended period of study.

Blood serum and twenty-four-hour urine samples were collected every seven days prior to and during PAS therapy and were analyzed for sodium and potassium by means of the flame photometer; lithium was used as an internal standard. The ranges of the serum sodium and potassium in 20 normal males were $144.5 \pm 5.44$ and $5.1 \pm 0.67$ mEq. per liter, and the twenty-four-hour urine sodium and potassium were $13.5 \pm 6.0$ and $6.0 \pm 1.5$ gm. per twenty-four hours.

## RESULTS

No appreciable effect of PAS upon the serum or twenty-four–hour urine potassium was found (table 1). The mean serum and twenty-four–hour urine potassium compared with control levels prior to PAS therapy revealed no significant difference.

Low initial serum sodiums of 121.30, 124.44, and 133.50 mEq. per liter were found in 3 patients (R. M., A. M., and G. E.) prior to therapy. Two of the patients (G. E. and A. M.) with hyponatremia continued to excrete sodium in the urine without correcting their serum sodium to normal for five to twelve weeks, respectively (table 2). With the exception of these 3 patients, the serum

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[2] Published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

TABLE 1

MEAN SERUM AND URINE POTASSIUM VALUES IN TEN PATIENTS RECEIVING PAS FOR TUBERCULOSIS

| PATIENT | SERUM POTASSIUM | | | URINE POTASSIUM | | |
|---|---|---|---|---|---|---|
| | Before* (mEq. per L.) | Number | During (mEq. per L.) | Before* (gm. per 24 hours) | Number | During (gm. per 24 hours) |
| R. A. | 5.7 | 5 | 4.7 ± 0.10 | 4.5 | 6 | 4.1 ± 1.20 |
| R. T. | 4.6 | 6 | 4.7 ± 0.56 | 6.1 | 5 | 6.6 ± 1.71 |
| R. A. | 4.7 | 6 | 4.6 ± 0.58 | 8.2 | 5 | 8.4 ± 0.99 |
| G. E. | 4.0 | 4 | 4.5 ± 0.36 | 5.7 | 7 | 5.9 ± 1.12 |
| R. M. | 3.5 | 7 | 4.3 ± 0.39 | 2.8 | 5 | 3.9 ± 0.48 |
| W. W. | 5.4 | 12 | 4.6 ± 0.35 | 6.5 | 12 | 7.6 ± 1.06 |
| A. M. | 4.7 | 9 | 4.1 ± 0.50 | 0.4 | 13 | 3.4 ± 1.62 |
| E. B.† | 4.6 | 11 | 4.5 ± 0.31 | 8.0 | 13 | 13.0 ± 2.83 |
| J. R. | 5.6 | 11 | 5.1 ± 0.31 | 6.9 | 11 | 7.2 ± 1.17 |
| M. V. | 4.3 | 10 | 4.5 ± 0.27 | 8.3 | 11 | 8.6 ± 1.60 |

* Average of 2 values.

† Febrile throughout the study.

TABLE 2

SERUM AND URINE SODIUM AND POTASSIUM IN TWO TUBERCULOUS SUBJECTS WITH HYPONATREMIA DURING PAS THERAPY

| WEEK | PATIENT G. R. | | | | PATIENT A. M. | | | |
|---|---|---|---|---|---|---|---|---|
| | Serum (mEq. per L.) | | Urine (gm. per 24 hours) | | Serum (mEq. per L.) | | Urine (gm. per 24 hours) | |
| | Na | K | Na | K | Na | K | Na | K |
| 1* | 133.5 | 4.0 | 18.5 | 5.7 | 124.4 | 4.7 | 5.9 | 0.4 |
| 2* | 133.5 | 4.1 | 17.6 | 5.6 | 124.5 | — | 8.1 | — |
| 3 | 133.1 | — | 9.9 | 6.7 | — | — | 9.6 | 0.9 |
| 4 | 136.4 | — | 18.0 | 5.8 | 115.3 | 4.5 | 15.8 | 0.8 |
| 5 | 126.6 | — | 11.5 | 4.8 | 124.0 | 4.7 | 16.4 | 1.3 |
| 6 | — | — | 17.6 | 6.9 | 133.5 | 4.6 | 15.3 | 3.5 |
| 7 | 141.3 | 4.0 | 19.7 | 7.5 | 125.60 | 4.3 | 21.0 | 3.4 |
| 8 | — | — | — | — | 132.6 | 3.5 | 15.3 | 2.9 |
| 9 | 150.8 | 4.7 | 9.9 | 5.2 | — | — | 16.5 | 3.6 |
| 10 | 150.8 | 4.7 | 12.5 | 4.6 | 127.7 | 4.3 | 16.5 | 3.6 |
| 11 | 152.2 | — | | | 104.6 | 3.7 | 13.2 | 5.5 |
| 12 | 153.5 | 4.7 | | | 122.3 | 4.0 | 15.8 | 3.2 |
| 13 | | | | | — | — | 15.7 | 5.3 |
| 14 | | | | | 142.7 | 3.7 | 12.0 | 5.8 |

* Controls.

and urine sodium levels were not significantly different prior to and during PAS therapy. The increased urine sodium excretion by all subjects during PAS therapy was accounted for by the additional 4 gm. of sodium present as the sodium salt of PAS (table 3).

The unusual development of a potassium deficiency in 2 patients during treatment with streptomycin and PAS was not included in the above study. No diarrhea was present.

### *Case Reports*

*Case R. B.* The patient was a 50-year-old white male clerk who was admitted on February 3, 1951, because of myalgia, fever, headache, and blurred vision of two weeks' duration. From December, 1950, he had noted difficulty in coordinating movements of the left hand, inability to recall recent events, and difficulty in mental concentration. He had previously been hospitalized from September, 1945, to September, 1948, for treatment of pulmonary and genital tuberculosis and his disease was considered inactive at the time of discharge.

Pertinent physical findings were a temperature of 102°F., blood pressure of 105 systolic, 70 diastolic in mm. of mercury, slight neck rigidity, and increased reflexes in the left upper

TABLE 3

MEAN SERUM AND URINE SODIUM VALUES IN TEN PATIENTS RECEIVING PAS FOR TUBERCULOSIS

| PATIENT | SERUM SODIUM | | | URINE SODIUM | | |
|---|---|---|---|---|---|---|
| | Before* (mEq. per L.) | Number | During (mEq. per L.) | Before* (gm. per 24 hours) | Number | During (gm. per 24 hours) |
| R. A. | 144.2 | 4 | 143.2 ± 1.54 | 5.2 | 6 | 8.6 ± 4.82 |
| R. T. | 147.8 | 6 | 145.2 ± 2.72 | 7.8 | 5 | 12.5 ± 1.42 |
| R. A. | 144.1 | 6 | 150.3 ± 1.41 | 16.9 | 5 | 18.2 ± 3.25 |
| G. E. | 133.5 | 8 | 143.1 ± 3.23 | 18.5 | 7 | 14.1 ± 4.14 |
| R. M. | 121.3 | 8 | 148.2 ± 1.53 | 9.9 | 6 | 12.1 ± 4.13 |
| W. W. | 149.6 | 8 | 150.1 ± 1.38 | 16.2 | 12 | 21.8 ± 3.22 |
| A. M. | 124.4 | 9 | 125.4 ± 3.48 | 5.9 | 13 | 15.0 ± 2.82 |
| E. B. | 144.3 | 7 | 146.6 ± 1.50 | 16.3 | 13 | 24.9 ± 7.52 |
| J. R. | 139.4 | 9 | 143.4 ± 1.94 | 12.9 | 11 | 19.4 ± 2.19 |
| M. V. | 145.5 | 9 | 146.3 ± 1.61 | 13.0 | 11 | 21.6 ± 2.52 |

* Average of two values.

and lower extremities. The hemogram was not remarkable except for an erythrocyte sedimentation rate of 39 mm. in one hour (Westergren). Urinalysis showed 1 plus albumin and 45 to 50 leukocytes per high-powered field. A serologic test for syphilis (Kahn) was negative. A roentgenogram of the chest revealed a right thoracoplasty with a diffuse reticular infiltrate in the left hilar area. Spinal tap showed slightly increased pressure. The cerebrospinal fluid contained 396 cells per cu. mm., of which 46 per cent were polymorphonuclear leukocytes, and 54 per cent lymphocytes; a total protein of 96 mg. per 100 ml.; sugar concentration of 28 mg. per 100 ml.; and chlorides, 117 mg. per 100 ml. Tubercle bacilli were cultured from the cerebrospinal fluid and urine.

Treatment consisted of bed rest; streptomycin, 1 gm. per day; oral PAS, 12 gm. per day; and intrathecal streptomycin, 50 mg. three times per week. Two months after admission the patient began to complain of nausea and gastric distress after taking PAS, with resultant anorexia which decreased his dietary intake to less than half his usual intake. Despite efforts to adjust dosage and palatability of the PAS, the symptoms progressed, and on April 30, 1951, he was noted to have marked muscle weakness, lethargy, extreme fatigue, and fever. Physical examination was not significant except for dehydration and a blood pressure of 90 systolic and 50 diastolic, and the patient was thought to have Addison's dis-

ease. Electrolyte studies on May 1, 1951, revealed a serum sodium of 137 mEq. per liter, potassium 2.58 mEq. per liter, chlorides 94.4 mEq. per liter, $CO_2$ 28 mEq. per liter, and a fasting glucose of 100 mg. per 100 ml.

Because of hypopotassemia, the patient was started on 2 gm. potassium chloride orally every four hours. Oral PAS was temporarily discontinued and PAS was given subcutaneously as a 1 per cent solution. The clinical response was remarkable, with a rapid disappearance of the symptoms within twelve hours. Electrocardiographic studies remained normal throughout. Subsequent electrolyte studies were noted (table 4).

*Case C. M.* The patient was a 53-year-old white male laborer who was admitted on May 5, 1950, with symptoms of dyspnea, orthopnea, cough, and swelling of the abdomen and lower extremities of two months' duration. Previous history indicated that he had developed

TABLE 4

CHEMICAL DETERMINATIONS OF THE BLOOD IN PATIENT R. B.

| DATE | Na (MEQ. PER L.) | K (MEQ. PER L.) | $CO_2$ (MEQ. PER L.) | Cl (MEQ. PER L.) | BUN (MG. PER 100 CC.) |
|---|---|---|---|---|---|
| 5/1 | 137.0 | 2.6 | 28.0 | 94.4 | 30 |
| 5/2 | 143.1 | 2.4 | 27.9 | 100.5 | 20 |
| 5/7 | — | 3.9 | 26.6 | 100.5 | 9.5 |
| 5/24 | 133.9 | 4.9 | 27.5 | 101.2 | 10 |
| 6/19 | 138.3 | 4.3 | 26.1 | 100.0 | — |

TABLE 5

OBSERVED SERUM ELECTROLYTES

| DATE | Na (mEq. per L.) | K (mEq. per L.) | Cl (mEq. per L.) |
|---|---|---|---|
| 4/24 | 126.1 | 2.4 | — |
| 4/25 | 123.9 | 3.0 | 68.7 |
| 4/27 | 130.5 | 4.1 | 80.7 |
| 5/4 | 141.3 | 4.5 | 90.3 |
| 5/23 | 137.0 | 4.7 | 99.5 |
| 6/28 | 141.3 | 4.4 | 111.2 |

a chronic right otitis media since December, 1948, and a swelling of the right scrotal contents in February, 1950.

Physical examination revealed a chronically ill male with distended neck veins, a chronic right otitis media, dullness over the left lung base with rales, a markedly enlarged area of cardiac dullness with distant heart tones, and a paradoxical pulse. The abdomen was distended with tenderness in the right upper quadrant and a palpable fluid wave. The right epididymis was enlarged, firm, and tender. There was pitting edema of both legs.

Vital capacity was 1.2 liters; venous pressure, 25 cm. of saline; and circulation time (arm to tongue) was 35 seconds with decholin. Hemogram and urinalysis were normal, and a serologic test for syphilis (Kahn) was negative. Roentgenograms of the chest revealed a left pleural effusion and greatly enlarged cardiac shadow.

The diagnosis of tuberculous pericarditis was confirmed by aspiration. Tubercle bacilli were cultured from the pericardial fluid and right ear. Treatment consisted of bed rest, 1 gm. of streptomycin per day, 12 gm. of oral PAS daily, and repeated pericardial aspirations to relieve tamponade. Despite slow improvement, the pericarditis became constrictive and pericardiectomy was done on March 28, 1951, under local anesthesia. The postoperative

course was remarkably uncomplicated but the patient subsequently noted difficulty in tolerating oral PAS, with associated nausea, anorexia, and marked reduction in the dietary intake. During the period of April 15 to 24, 1951, muscle weakness, listlessness, and mental confusion increased. Electrolyte studies on April 24, 1951, revealed a serum sodium of 126.1 mEq. per liter and potassium of 2.4 mEq. per liter. He was given 2 gm. of potassium chloride orally every four hours and intravenous saline, with marked improvement in his mental state and muscular weakness. No electrocardiographic abnormalities attributable to hypopotassemia were detected during this episode. The electrolyte studies indicated the rapid reversal of his hyponatremia and hypopotassemia (table 5).

## DISCUSSION

A potassium deficiency may be associated with (*a*) an inadequate dietary intake (3), (*b*) an increased loss in the gastrointestinal or genitourinary tracts (4, 6, 7), (*c*) overtreatment with drugs (5), or (*d*) metabolic states (8, 9).

The usual complication of a potassium deficiency associated with oral PAS therapy may be related to an increased excretion of potassium and to a dietary insufficiency or both. The normal adult daily ingests and excretes 3 to 4 gm. of potassium (75 to 100 mEq.) (10, 11). Fasting adults receiving water may excrete 1 to 3 gm. (25 to 75 mEq.) of potassium per day (12, 13). The present study did not reveal an increased excretion of potassium in the urine of those patients taking PAS and on an adequate dietary intake. Oral PAS in doses of more than 8 gm. per day frequently will cause gastric irritation with resultant nausea, vomiting, and anorexia leading to an inadequate dietary intake. The continued renal excretion of potassium despite a markedly diminished exogenous potassium intake may well account for the potassium deficiency observed in the 2 cases here reported as well as in previous studies (1, 2). Nevertheless, a potassium deficiency may be secondary to oral PAS irritation on the gastrointestinal tract without apparent diarrhea.

A sodium deficiency in patients with active pulmonary tuberculosis has been described by Winkler and Crankshaw (14), Westover and associates (15), and Sims and co-workers (16). The hyponatremia found in 3 patients in the present series may be secondary to severe disease associated with malnutrition and is not associated with PAS therapy. Two patients, G. E. and A. M., were unable to correct the serum sodium to normal for five to twelve weeks, respectively, although they continued to excrete 9 to 19 gm. of sodium in the urine per day. Only after clinical improvement did the serum sodium gradually return to normal. A disturbed renal electrolyte control in primary non-renal disease (17) or a chronic reduction of cellular osmolarity (16), or both, may be the primary deficit.

## SUMMARY

A study of serum and urine sodium and potassium prior to and during oral para-aminosalicylic therapy in 10 patients indicated no significant difference in the values in the two periods. Ingested PAS intrinsically has no apparent effect upon sodium or potassium balance. The effects of oral PAS influencing the dietary intake or gastrointestinal irritation without apparent diarrhea, or both, may account for the hypopotassemia and related clinical signs and symptoms observed

in the 2 cases presented in this study. Both responded quickly and effectively to replacement with oral potassium as potassium chloride. Three cases of hyponatremia occurring during active pulmonary tuberculosis were noted and electrolyte studies are reported revealing the slow return to normal values with clinical improvement.

SUMARIO

*Hipopotasemia e Hiponatremia en la Tuberculosis Pulmonar Durante el Tratamiento*

El estudio del sodio y del potasio en el suero y en la orina de 10 enfermos antes y después de la terapéutica oral con ácido para-aminosalicílico (PAS) no indicó mayor diferencia. El PAS ingerido no ejerce intrínsecamente efecto aparente sobre el equilibrio del sodio y del potasio. Los efectos del PAS por vía oral, ya afectando la ingestión dietética o produciendo irritación gastrointestinal sin diarrea manifiesta, pueden explicar la hipopotasemia y signos y síntomas afines observados en los 2 casos presentados en este estudio. Ambos respondieron rápida y eficazmente a la substitución oral del potasio en forma de cloruro potásico. Se notaron 3 casos de hiponatremia sobrevenida durante la tuberculosis pulmonar activa, y los estudios de los electrolitos revelaron un retorno lento a cifras normales al ocurrir mejoría clínica.

RESUME

*Hypopotassémie et hyponatrémie au cours du traitement de la tuberculose pulmonaire*

Une étude du sodium et du potassium sérique et urinaire effectuée chez 10 malades, avant et pendant le traitement oral par l'acide para-aminosalicylique (PAS), n'a permis d'observer aucune différence. Le PAS ingéré n'a pas d'action apparente propre sur l'équilibre du sodium et du potassium. Les effets du PAS oral, par son action sur l'apport alimentaire ou par l'irritation gastro-intestinale qu'il provoque, sans phénomènes diarrhéiques apparents, ou par ces deux actions, peuvent expliquer l'hypopotassémie et les signes cliniques relatés, ainsi que les symptômes observés dans les deux cas présentés dans cette étude. Tous les deux ont réagi rapidement et efficacement à la thérapeutique de remplacement par le potassium oral, sous la forme de chlorure de potassium. Il a été observé 3 cas d'hyponatrémie au cours de la tuberculose pulmonaire en activité et l'étude des électrolytes, qui est rapportée, montre le retour lent aux valeurs normales en même temps que l'amélioration clinique.

*Acknowledgments*

The writers wish to express thanks to Dr. William B. Tucker for his helpful suggestions and criticisms. They also wish to thank Miss Muriel Vanderbilt for technical assistance in carrying out the laboratory procedures for the above study.

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# ADRENOCORTICAL FUNCTION IN PULMONARY TUBERCULOSIS[1]

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## INTRODUCTION

There are a number of findings and symptoms in pulmonary tuberculosis suggestive of adrenocortical hypofunction. Hypochloremia, hyponatremia, favorable response to adrenal cortical extract, decreased ability to produce a normal water diuresis, gastrointestinal upsets, debilitation, and anorexia are not infrequent findings or have been described in occasional instances. A paradoxical situation exists in that approximately 50 per cent of patients with Addison's disease show tuberculosis of the adrenal gland and yet Addison's disease itself is rarely seen in a tuberculosis hospital. Similar examples are tuberculosis of the eye and of the skin where reactivation of a dormant foci of infection in an organ may occur following lymphogenous or hematogenous spread from the original primary foci.

### *Review of Literature*

Aronson (1), in autopsies of 579 persons who died in army hospitals from 1941 to 1945 of acute progressive tuberculosis (average of 5.8 months from diagnosis to death), found that 25.3 per cent showed tuberculosis of the adrenal gland. A high percentage (36.9 per cent) had tuberculous meningitis; and, in 408 of the 579 cases, generalized miliary tuberculosis was present. In a recent report on 500 autopsies of sanatorium patients who died of pulmonary tuberculosis, Davenport and Greenleaf (2) found that 14 per cent showed tuberculosis of the adrenal glands, but stated that "the involvement was not extensive."

In fifteen years of exclusive sanatorium practice, Bronfin and Guttman (3) encountered in approximately 4,500 patients with pulmonary tuberculosis only 2 cases of Addison's disease due to tuberculosis of the adrenal gland. They quote Minor (4) as not observing a single case in a period of thirty-five years. In the present writers' sanatorium no case has been diagnosed in more than 10,000 admissions.

In 1940, Thorn and his group (5) studied electrolyte changes in pulmonary tuberculosis, with particular reference to the adrenal cortical function. They noted that:

> patients with pulmonary tuberculosis and extensive nontuberculous pulmonary disease were observed to have a striking decrease in serum chloride concentration which paralleled the reduction in vital capacity.

Recently, Kolmer and his associates (6) studied 92 pulmonary tuberculosis patients and noted a significant decrease in both chlorides and total base concentration in the serum but a normal carbon dioxide and pH. They noted no striking relationship between degree of tuberculous involvement and the level of "acid-base condition," but did note that those patients with higher degrees of fever had the lowest levels of serum chloride and total base. Sims and his associates (7) studied 10 tuberculous patients with hyponatremia, all with far advanced tuberculous lesions. The adrenal glands were normal in 6 patients who were examined post mortem. The Thorn test was done on 6 patients and was found to be normal.

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Casas and Aguiar (8) performed the Robinson-Kepler-Power (R-K-P) test for adrenal insufficiency on 32 patients with pulmonary tuberculosis and 13 showed a positive test. They treated the positive patients with Percorten and Corticon, and the R-K-P test returned to normal in all but one case. In 1949, Cheney (9) successfully treated a patient with pulmonary tuberculosis who developed Addison's disease with eschatin and streptomycin.

Numerous articles appeared in the older literature of pulmonary tuberculosis on the diagnostic and prognostic significance of hypotension. Stivelman (10) quotes Marfan, considered to be the earliest to study the problem, as saying 97 per cent of all tuberculous patients had hypotension. Fishberg (11) stated "it has been found that a low blood-pressure is an almost constant characteristic of the very early stage of phthisis." Morlock and Horton (12) reported on blood pressures of 346 patients with proved tuberculosis of the kidneys and found that 76 per cent had normal blood pressure. Masten (13), in 230 cases, concluded that the average blood pressure was normal but that hypotension, present on admission to the hospital, which did not improve with treatment was associated with a poor prognosis.

With the recent advent of adrenocortical hormones, eosinophils have assumed a very important role. When 25 mg. of adrenocorticotropin (14) are administered intramuscularly (Thorn test), the eosinophils in a patient with normal adrenal glands drop 50 per cent or more in a four-hour period from their normal circulating level of approximately 200 cells per cu. mm. by direct count. This is considered to be the result of increased output of the 11–17 oxysteroids of which cortisone is a prototype. Instead of using adrenocorticotropin, 0.3 cc. of epinephrine (1:1,000) (15) may be used. As epinephrine is mediated through the pituitary gland, this is a test of both pituitary function and adrenal cortical reserve.

With the above brief review of the literature in mind, it was decided first to see if tuberculous patients in this hospital showed hypotension. Moreover, as the eosinophile test represented a simple inexpensive test for adrenal cortical function, it was decided to apply this test to the population of a large tuberculosis sanatorium to determine if any correlation existed between adrenal cortical reserve and active pulmonary tuberculosis.

## METHODS

All patients had pulmonary tuberculosis proved bacteriologically by smear or culture. All of the patients were white, and all the various regimens of therapy for tuberculosis were in effect. With few exceptions, the group studied for blood pressure and those receiving the Thorn test are identical.

### *Blood Pressure*

The pressures were all taken with the patient lying in bed during the regular afternoon rest hour. The pressures were all taken on the right arm with the same Tyco sphymomanometer for three consecutive days, and an average was made of the results on 229 cases (table 1).

### *Thorn Test*

The patients were instructed to take no food or liquids (except water) by mouth, and parenteral medications were withheld from midnight prior to the test until after the test was completed at noon of the next day. At 7:30 a.m. the blood was taken from the finger

directly into a leukocyte pipette, using the method of Pilot (16); this was shaken gently for thirty seconds. Then 0.3 mg. of epinephrine was injected subcutaneously and another specimen of blood taken four hours later directly into a leukocyte pipette. Previous experiments with this diluting fluid showed that the cells reached maximal number in thirty to sixty minutes and remained stable three to five hours. The counts were made within one or two hours in a hemacytometer; two chambers were counted and an average made of the results.

TABLE 1

BLOOD PRESSURE DETERMINATIONS IN PULMONARY TUBERCULOSIS

| | MINIMAL | | MODERATELY ADVANCED | | FAR ADVANCED | |
|---|---|---|---|---|---|---|
| | Systolic | Diastolic | Systolic | Diastolic | Systolic | Diastolic |
| **By Sex** | | | | | | |
| Female | 115 | 74 | 122 | 79 | 118 | 79 |
| Range | 123–101 | 74–65 | 186–100 | 95–61 | 165–80 | 107–58 |
| Number of cases | 12 | | 47 | | 60 | |
| Male | 128 | 79 | 125 | 80 | 116 | 78 |
| Range | 134–123 | 88–77 | 157–102 | 97–68 | 157–94 | 89–73 |
| Number of cases | 4 | | 39 | | 67 | |
| Average | 118 | 75 | 123 | 80 | 117 | 79 |
| Range | 134–101 | 88–65 | 186–100 | 95–61 | 165–80 | 107–58 |
| Number of cases | 16 | | 86 | | 127 | |
| **By Age** | | | | | | |
| 13 to 20 years | 121 | 77 | 120 | 76 | 119 | 78 |
| Range | 134–108 | 88–68 | 133–108 | 77–70 | 148–94 | 105–75 |
| Number of cases | 9 | | 5 | | 9 | |
| 21 to 40 years | 116 | 75 | 122 | 79 | 119 | 79 |
| Range | 128–106 | 80–75 | 157–100 | 97–61 | 165–94 | 107–73 |
| Number of cases | 6 | | 58 | | 68 | |
| 41 to 65 years | 101 | 65 | 127 | 81 | 118 | 79 |
| Range | | | 186–102 | 95–68 | 159–91 | 113–58 |
| Number of cases | 1 | | 23 | | 50 | |

## RESULTS

### *Blood Pressure*

Eighty-seven per cent of the patients had a systolic pressure between 100 and 140 in mm. of mercury. Five per cent had systolic pressures of 100 mm. or below and 8 per cent had systolic pressures above 140 mm. Nineteen of the 229 patients died within the year after the pressures were taken, 4 of whom had a systolic pressure of 100 mm. or below. Of the number who died, one was in the 13- to 20-year age group; her blood pressure was 94 systolic and 75 diastolic in mm. of mercury. In the 21- to 40-year age group, 8 died with an average pres-

sure of 110 systolic and 77 diastolic and a range of 121 systolic and 79 diastolic to 101 systolic and 71 diastolic in mm. of mercury. In the 41- to 65-year age group, 10 died with an average pressure of 110 systolic and 70 diastolic and a range of 157 systolic and 89 diastolic to 91 systolic and 58 diastolic in mm. of mercury.

### *Thorn Test*

It was the original intention to do the Thorn test on patients who showed hypotension; however, since the tuberculous patients did not have hypotension, it was decided to do the less expensive epinephrine test on all ward patients.

The average fasting count was 227 circulating eosinophils per cu. mm. (table 2). There appeared to be no significant difference in the fasting count as related

TABLE 2

THORN TEST IN PULMONARY TUBERCULOSIS

| CLASSIFICATION | NUMBER OF CASES | FASTING COUNT | FOUR HOURS AFTER EPINEPHRINE | PERCENTAGE DROP |
|---|---|---|---|---|
| Minimal | 16 | 218 | 64 | 71 |
| Moderately advanced | 86 | 250 | 77 | 69 |
| Far advanced | 135 | 213 | 59 | 72 |
| Average | 237 | 227 | 66 | 71 |
| Repeat cases done without prior knowledge of patient (all having previously shown less than 50 per cent drop) | 69 | 238 | 87 | 63 |
| Patients with temperature above 99°F. | 24 | 173 | 48 | 72 |
| Patients with systolic blood pressure 100 mm. or below | 15 | 230 | 98 | 57 |

to the stage of disease. The percentage drop averaged 71, which is the usual response. Seventy-one cases failed to show the 50 per cent or more drop in circulating eosinophils after epinephrine stimulation. Many patients were unusually apprehensive about the test, even to the extent of gross tremors before epinephrine was administered. Due to this emotional element, the tests were repeated without prior knowledge by the patient and 62 of 69 were normal; 2 of the 71 patients refused to allow a second test. The percentage drop was 63, including those who did not have an adequate response.

*Fever:* Twenty-four cases had fever of 99°F. or more. These patients showed fasting counts of 173 eosinophils in contrast to 227 for the entire group, but the average percentage drop was 72 per cent. The least percentage drop occurred in 15 patients who had a systolic pressure of 100 mm. or less; their fasting count being 230 cells with 98 cells four hours later for a drop of 57 per cent.

*Antimicrobial therapy:* Forty-four patients were receiving dihydrostreptomycin on a regimen of 1 gm. every third day and para-aminosalicylic acid daily,

and at the time of this test had had varying amounts of dihydrostreptomycin up to a total dosage of 40 gm. Although eosinophilia has been described in patients on streptomycin therapy (17), the average fasting eosinophile count of these patients on dihydrostreptomycin was 216 compared with 227 cells for all patients. With eosinophilia arbitrarily designated as the presence of 500 or more cells, only 20 of 237 patients showed a fasting count above this level. Of the 44 patients receiving dihydrostreptomycin, 4 had eosinophilia. Each of these 4 had had at least 35 gm. of dihydrostreptomycin. The highest eosinophile count was in a patient not receiving dihydrostreptomycin (1,265 cells per cu. mm.).

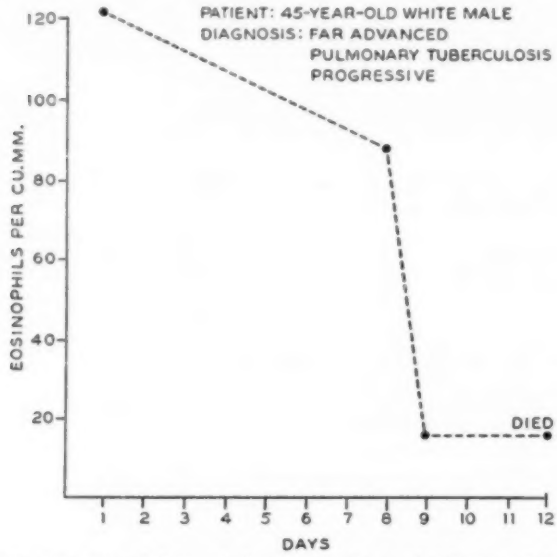


FIG. 1. Fasting eosinophile counts in a terminal case of pulmonary tuberculosis

*Terminal illness:* Five patients with progressive tuberculosis were followed with fasting counts for varying periods up to death. On basis of the last test before death, one had no circulating eosinophils at all on the day of death, 2 others had zero and six cells the day prior to death, and the other 2 had eleven and zero cells three and four days, respectively, before death (figure 1).

*Surgical procedures:* Several patients were followed while undergoing thoracoplasty and showed a normal eosinophile response (18) with a reduction to five cells or less several hours after surgery and a gradual return to normal within two to three days postoperatively (figure 2). Similar curves were obtained for subjects undergoing delivery, extrapleural plombage, and surgery for intestinal obstruction. At the time of the third-stage thoracoplasty, done within four weeks of the first, patients showed a similar response but required four to five days for the eosinophile count to return to normal.

*Entire series:* In this series of 237 patients, 228 persons responded with a decrease in the eosinophile count by 50 per cent or more to a dose of 0.3 mg. of 1:1,000 epinephrine in the four-hour test period. This includes the results in 62 patients on whom the test was repeated. Seven subjects demonstrated poor response even to the second test dose. Two patients refused a second injection of epinephrine. One of these 2 had an initial fasting count of zero (which was repeated with similar results on several occasions), and so may be considered to have had adequate function even though she died shortly thereafter. The 7 who had a poor response after two tests were given part I of the Robinson-Kepler-Power test, and all but one showed a normal water diuresis.

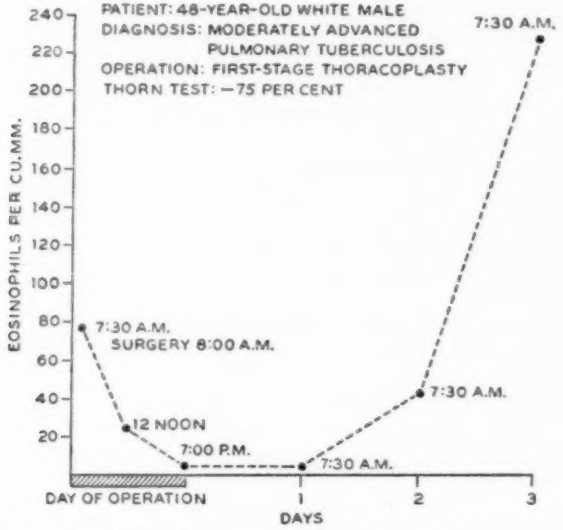


FIG. 2. Fasting eosinophile response following surgery in a case of pulmonary tuberculosis

## DISCUSSION

Hypochloremia, hyponatremia, favorable response to adrenal cortical extract, decreased ability to produce a normal water diuresis, gastrointestinal upsets, debilitation, and anorexia are findings which have been described in pulmonary tuberculosis. The relation of these findings to possible hypoadrenocortical reserve in tuberculosis has been discussed in a review of the literature.

Blood pressure observations were done on 229 cases of pulmonary tuberculosis. The pressures were not obtained in order to aid prognosis or to study blood pressure per se in tuberculous patients. Nevertheless, the findings in this limited number of cases suggest that the average patient with pulmonary tuberculosis does not have hypotension in any stage of the disease. In those patients who do have a systolic blood pressure of 100 mm. or below, however, the prognosis is

not as good as in those with pressures above 100 mm. systolic. These findings are somewhat similar to those of Masten (13).

The Thorn test was carried out on 237 patients, with only 9 patients failing to show a 50 per cent or more decrease in circulating eosinophils. Two patients refused another test; a repeat test on the remaining 7 patients continued to show low adrenal cortical reserve, but 6 of the 7 patients were able to produce a normal water diuresis to part I of the Robinson-Kepler-Power test.

Some patients developed sufficient so-called stress when informed in advance of the Thorn test to invalidate the results. Recant and his associates (15) describe a similar situation in rats where the mere taking of blood from the tail without further stimulation causes an average drop of 78 per cent in circulating eosinophils. These investigators further state that "the demonstration of a relative refractoriness of the eosinopenic response to epinephrine with a normal response to cortical extract at this time suggests that a time lag exists between hormonal discharge and resynthesis." In other words, the patients, through tension and fright (alarm reaction), were producing their own self-induced Thorn test. A repeat test without prior knowledge by the patient showed normal adrenocortical reserve in 62 of 69 patients.

Contrary to some reports of eosinophilia (by differential count) in patients receiving streptomycin, only 4 of 44 patients in this series receiving dihydrostreptomycin showed an eosinophilia, using the direct eosinophile count. This difference may possibly be explained by the use of dihydrostreptomycin or on the basis of intermittent instead of daily therapy.

Five patients were followed with fasting eosinophile counts for varying periods up to the day of death. On the basis of the last test before death, the highest count was 17 eosinophils per cu. mm. and the lowest was zero. Forsham and Thorn (19) believe that "the finding of fewer than 50 eosinophils per cu. mm. of blood rules out the diagnosis of primary adrenal cortical insufficiency."

Tuberculous patients show a normal eosinophile response to the stimulus of surgical procedures.

## SUMMARY

Blood pressure observations on 229 tuberculous patients under resting conditions for three consecutive days showed a normal range in 87 per cent of the cases.

The Thorn test was carried out on 237 patients. Seventy-one patients failed to show a 50 per cent or more decrease in circulating eosinophils. A repeat test in 69 of these 71 patients, without their prior knowledge, showed adequate adrenocortical reserve in 62 patients. Of the remaining 7 patients, 6 were able to produce a normal water diuresis to part I of the Robinson-Kepler-Power test.

Some patients developed sufficient stress, when informed in advance of the Thorn test, to invalidate the results. A repeat test, without informing the patient in advance, showed normal adrenocortical reserve in 62 of 69 patients.

Tuberculous patients show a normal eosinophile response to the stimulus of surgical procedures.

On the basis of these results, it is believed that the average case of pulmonary tuberculosis had adequate adrenocortical reserve.

SUMARIO

*La Función Adrenocortical en la Tuberculosis Pulmonar*

Las observaciones de la tensión sanguínea en 229 tuberculosos en estado de reposo durante tres días consecutivos revelaron cifras normales en 87 por ciento de los casos.

En 237, se ejecutó la prueba de Thorn. De ellos, sólo 71 dejaron de mostrar una disminución de 50 por ciento o más en los eosinófilos en la circulación. Otra prueba verificada en 69 de dichos 71 enfermos, sin avisarlos de antemano, reveló adecuada reserva adrenocortical en 62. De los 7 restantes, 6 acusaron una hidrodiuresis normal en la Parte I de la prueba de Robinson-Kepler-Power.

Algunos sujetos desplegaron suficiente tensión, al ser avisados de antemano que iba a ejecutárseles la prueba de Thorn, para invalidar el resultado. Otra prueba, ejecutada sin poner sobre aviso al enfermo, mostró reserva adrenocortical normal en 62 de 69 enfermos.

Los tuberculosos revelan una respuesta eosinofílica normal a la cirugía.

A base de esos resultados, opinan los AA. que la mayoría de los casos de tuberculosis pulmonar poseen una reserva adrenocortical adecuada.

RESUME

*Fonction cortico-surrénale dans la tuberculose pulmonaire*

Des observations sur la tension artérielle chez 229 tuberculeux, mis au repos pendant trois jours consécutifs, ont montré des valeurs normales dans 87 pour 100 des cas.

Le test de Thorn a été effectué chez 237 malades. Chez 71 malades la réduction de 50% ou plus du nombre des éosinophiles de la circulation n'a pu être décelée. La répétition du test effectuée chez 69 de ces 71 malades, et à leur insu, a montré une réserve adéquate de la fonction cortico-surrénale chez 62 malades. Parmi les 7 derniers malades, 6 ont été en mesure de réagir par une diurèse aqueuse normale à la première partie du test de Robinson-Kepler-Power.

Le "stress" a été suffisamment marqué chez quelques malades ayant été informés d'avance du test de Thorn, pour en vicier les résultats. Un test renouvelé, sans que le patient en soit informé d'avance, a permis d'observer une réserve cortico-surrénale normale chez 62 malades sur 69.

Dans les cas chirurgicaux, le malade tuberculeux présente une réaction éosinophilique normale.

Sur la base de ces résultats, les auteurs pensent que le cas moyen de tuberculose pulmonaire dispose d'une réserve cortico-surrénale adéquate.

*Acknowledgment*

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# *Case Reports*

## DISTURBANCE IN CARBOHYDRATE METABOLISM ASSOCIATED WITH AMITHIOZONE THERAPY[1,2]

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(Received for publication April 7, 1952)

### INTRODUCTION

In the never-ending quest to find effective drugs for the treatment of tuberculosis, amithiozone has been extensively studied both in Europe and the United States (1). Because amithiozone or its derivatives may continue to be useful in streptomycin-resistant cases and also may have a primary use in those cases of tuberculosis in which it may be desired to preserve streptomycin activity for future use, it is natural to become concerned with all the manifestations of its toxicity. Among the manifestations of toxicity reported in the literature, no mention has been made regarding the effects of amithiozone on the latent diabetic or diabetic state except in a previous report of two cases by Skavlem (2). Two cases of pulmonary tuberculosis treated with amithiozone are presented, one showing the development of diabetes, the other showing aggravation of an otherwise well-controlled diabetes.

### CASE REPORTS

*Case 1.* G. B. M. was a 32-year-old male farm laborer of Mexican lineage who has been hospitalized at Birmingham Veterans Administration Hospital and Long Beach Veterans Administration Hospital since December 27, 1949. He had far advanced bilateral fibro-exudative pulmonary tuberculosis with bilateral cavitation. His sputum was persistently positive for *M. tuberculosis*, and he had tuberculous laryngitis. The past history and family history are not relevant except that the patient admittedly was a heavy drinker of alcoholic beverages for many years. His right arm was amputated because of a wound. Except for tuberculous laryngitis, fever, and toxemia, the physical examination was not relevant.

After one week of graduated increase in dosage, 100 mg. of amithiozone twice daily was begun on January 19, 1950, concomitantly with 1 gm. of streptomycin daily. Pneumoperitoneum was started on March 8, 1950, and was continued to date. Streptomycin was discontinued on May 17, 1950, but amithiozone was continued, 50 mg. twice daily until November 20, 1950. By the end of the streptomycin course on May 17, 1950, the patient had become afebrile, gained twelve pounds, and most of the exudative element of his disease cleared; nevertheless, one large cavity remained in each lung. The laryngeal tuberculosis had healed. Clinically, no toxic effects were noted from either the streptomycin or the amithiozone.

Serial bromsulfalein tests done between January 24 and May 10, 1950, gave results of 24 per cent, 3 per cent, 20 per cent, 47 per cent, and 41 per cent retention of dye. Serial determinations of cephalin flocculation, thymol turbidity, van den Bergh, Congo red tests, blood urea nitrogen, serum albumin, and globulin yielded values which were essentially

[1] From the Pulmonary Disease Service, Veterans Administration Hospital, Long Beach, California.

[2] Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

within normal limits throughout his hospitalization. Phenolsulfonphthalein tests showed moderate impairment of renal function throughout hospitalization.

The urinalyses done before the beginning of streptomycin-amithiozone therapy showed normal findings. The blood sugar was not determined. With an onset March 20, 1950, however, at which time the patient was on amithiozone therapy, there followed persistent glycosuria (frequently 3 plus or 4 plus) without acetone. Fasting blood sugars as high as 166 mg. per 100 cc. of venous whole blood were obtained. There was occasionally mild albuminuria. At this point the possible causal relationship of the amithiozone therapy to the glycosuria and hyperglycemia was investigated and the possibility of an adrenal dysfunction was evaluated. In order to exclude the possibility of other reducing substances which may have formed in the metabolism of the amithiozone, fermentation studies and intravenous insulin studies were carried out which indicated that the glycosuria and hyperglycemia were due to glucose.

TABLE 1

LABORATORY DATA IN CASE 1

| LABORATORY TESTS | COLUMN 1 BEFORE REINSTITUTION OF AMITHIOZONE | COLUMN 2 AMITHIOZONE REINSTITUTED | COLUMN 3 AFTER AMITHIOZONE WAS DISCONTINUED |
|---|---|---|---|
| Urine sugar daily | Negative to occasional trace | 1+ for sugar; acetone negative | Negative to occasional trace; acetone negative |
| 24-hour quantitative urine sugar | Negative | 6.5 gm. (0.5 per cent) | Negative |
| Fasting blood sugar per 100 ml. of whole blood | 120 mg. | 154 mg. | 138 mg. |
| 24-hour uric acid excretion | 33 mg. per 100 ml. or 0.7 gm. per total volume | 0.5 gm. per total volume of 1,600 ml. | 48 mg. per 100 ml. or 0.63 gm. per total volume of 1,310 ml. |
| Uropepsin excretion | 10 units per hour | 7 units per hour | 15 units per hour |
| 17-ketosteroid excretion | 23 mg. per 24 hours; volume, 2,400 ml. | 9 mg. per 24 hours; volume, 1,360 ml. | 17 mg. per 24 hours; volume, 2,120 ml. |

After the amithiozone was discontinued on November 20, 1950, the patient's diabetes showed moderate improvement. The urine sugar was estimated at 1 plus to 3 plus, and the fasting blood sugar dropped to 123 mg. per 100 ml. of whole blood. On January 3, 1951, the patient was placed on a 2,000-calorie diet which consisted of 205 gm. of carbohydrate, 90 gm. of protein, and 95 gm. of fat. This diet was maintained throughout the study. Beginning January 7, 1951, the urine sugar, determined before breakfast and three hours after meals, varied from negative to an occasional trace; the blood sugars were approximately 132 mg. per 100 ml. of whole blood. On October 18, 1951, the laboratory data were obtained and are shown in column 1, table 1.

The patient was again placed on 100 mg. of amithiozone daily on November 20, 1951; by December 2, 1951, he again showed disturbance in carbohydrate metabolism with glycosuria approximately 1 plus in four daily specimens; acetone was absent. Chemical data were then obtained on December 27, 1951, and are shown in column 2, table 1.

On December 27, 1951, the amithiozone was again discontinued. By January 22, 1952, the diabetes had improved; the daily urine examinations were either negative or revealed an occasional trace of sugar without acetone. The laboratory findings obtained on January 15, 1952, are shown in column 3, table 1.

*Case 2.* M. M. S., admitted November 14, 1950, was a 46-year-old white male retired Navy communications man who had had diabetes mellitus since 1944. His diabetes was well controlled for more than a year prior to admission by 30 units of protamine zinc insulin daily and a 2,200-calorie diet which consisted of 221 gm. of carbohydrate, 88 gm. of protein, and 105 gm. of fat. Pulmonary tuberculosis was diagnosed in October, 1949. Physical examination was unrevealing. One brother had diabetes. The patient's past history was not pertinent except that he had been a heavy beer drinker for many years. The physical findings were not relevant.

Pneumoperitoneum was started November 3, 1949, for far advanced fibro-exudative pulmonary tuberculosis with bilateral cavitation, and is being maintained to date. Concurrently with the beginning of pneumoperitoneum, 1 gm. of streptomycin and an undetermined amount of PAS were given daily for 120 days. Left phrenic crushes were done in February and April, 1950.

TABLE 2

LABORATORY DATA IN CASE 2

| LABORATORY TESTS | COLUMN 1 BEFORE REINSTITUTION OF AMITHIOZONE | COLUMN 2 AMITHIOZONE REINSTITUTED | COLUMN 3 AFTER AMITHIOZONE WAS DISCONTINUED |
|---|---|---|---|
| Urine sugar daily | Negative | 4+ for sugar; acetone negative | Negative to trace; acetone negative |
| 24-hour quantitative urine sugar | Negative | 27 gm. (0.75 per cent) | 7.0 gm. (0.25 per cent) |
| Fasting blood sugar per 100 ml. of whole blood | 82 mg. | 194 mg. | 138 mg. |
| 24-hour uric acid excretion | 23 mg. per 100 ml. or 0.56 gm. per total volume of 2,420 ml. | 1.0 gm. per total volume of 3,600 ml. | 16 mg. per 100 ml. or 0.45 gm. per total volume of 2,820 ml. |
| Uropepsin excretion | 90 units per hour | 107.2 units per hour | 41 units per hour |
| 17-ketosteroid excretion | 10 mg. per 24 hours; volume, 1,950 ml. | 18 mg. per 24 hours; volume, 3,425 ml. | 10 mg. per 24 hours; volume, 3,100 ml. |

Bromsulfalein, cephalin flocculation, thymol turbidity, and blood urea tests were normal.

Urine samples throughout his hospitalization contained 1 plus to 2 plus sugar. On March 22, 1951, the administration of 1 gm. of streptomycin every other day for ten doses and amithiozone, 100 mg. daily, was begun and by March 23, 1951, the urines became 3 plus and 4 plus. No acetone or albumin was present. Fasting blood sugars were 160 mg. per 100 ml. The patient's insulin requirement increased to 40 units of protamine zinc insulin and 15 units of regular insulin, and the diet was reduced to 2,000 calories in order to control him as before amithiozone therapy.

On September 18, 1951, amithiozone was discontinued. On September 26, 1951, the patient suffered a mild insulin reaction, at which time his fasting blood sugar was 76 mg. per 100 ml. By October 19, 1951, the patient was again well controlled on 30 units of protamine zinc insulin daily and a 2,000-calorie diet, both of which were maintained throughout the study. Urine examinations became negative for sugar, the fasting blood sugars were approximately 138 mg. per 100 ml. Other laboratory findings are shown in column 1, table 2.

On October 19, 1951, 100 mg. amithiozone daily was again started; by November 14, 1951, urine examinations became 4 plus for sugar but remained negative for acetone. The laboratory data then obtained are shown in column 2, table 2.

Amithiozone was stopped on November 20, 1951. By December 7, 1951, the urine examinations were either negative or revealed a trace of sugar without acetone. Other laboratory reports at that time are indicated in column 3, table 2.

## COMMENT

Two cases of pulmonary tuberculosis in which the patients showed demonstrable impairment of carbohydrate metabolism associated with prolonged administration of amithiozone are reported. It is believed that the impairment of carbohydrate metabolism noted in these two patients was directly associated with the effects of amithiozone because one patient with no familial or personal history of diabetes developed diabetes, while the other patient with well-controlled diabetes had aggravation of the diabetic state. Both were well controlled on cessation of amithiozone therapy, became worse on reinstitution of amithiozone, and again became well controlled when the amithiozone was discontinued.

That the glycosuria and hyperglycemia were due to glucose and not due to chemical compounds associated with the metabolism of amithiozone was proved by fermentation studies of the blood and urine and by noting a significant drop in blood sugar upon the injection of intravenous insulin in one patient.

The mechanism whereby amithiozone produced impairment of carbohydrate metabolism is not known. Moreover, although one patient had impaired liver and renal function even before the amithiozone was started, the other patient had normal liver and renal function throughout the study. Therefore, it may be surmised that neither organ was necessarily responsible for the glycosuria and hyperglycemia. Although exhaustive tests were not done, it is believed that the adrenals were not involved, since uric acid, 17-ketosteroid, and uropepsin excretion studies were not significantly changed in either case during the courses of amithiozone.

Bünger (3) cites four cases of tuberculous meningitis treated with streptomycin, PAS, and amithiozone. These were associated with various endocrine disturbances, and two of them had disturbances of carbohydrate metabolism. It is his belief that the endocrine disturbances may have been associated with vascular processes resulting from the tuberculous meningitis. In the two cases herein reported, however, it is believed that the disturbance in carbohydrate metabolism may have resulted from amithiozone therapy alone.

## SUMMARY

Two cases of pulmonary tuberculosis in which the patients developed disturbance in carbohydrate metabolism under prolonged amithiozone therapy are reported. One developed diabetes, and the other had an aggravation of his previously well-controlled diabetic state.

## SUMARIO

*Trastorno del Metabolismo Hidrocarbonado Enlazado con la Amitiozonoterapia*

En 2 tuberculosos pulmonares se presentaron trastornos del metabolismo hidrocarbonado durante un tratamiento prolongado con amitiozona. Uno manifestó diabetes y el otro experimentó una agravación de un estado diabético previamente cohibido.

## RESUME

*Troubles du métabolisme des hydrates de carbone associés à la thérapie par l'Amithiozone*

Deux malades atteints de tuberculose pulmonaire, qui ont présenté des troubles du métabolisme des hydrates de carbone sous l'influence de la thérapie par l'amithiozone, sont

l'objet d'une communication. Un des malades a été affecté de diabète et l'autre a subi une aggravation de son état diabétique, précédemment bien contrôlé.

## REFERENCES

(1) MERTENS, A., AND BUNGE, R.: The present status of the chemotherapy of tuberculosis with Conteben, a substance of the thiosemicarbazone series, Am. Rev. Tuberc., 1950, *61*, 20.

(2) SKAVLEM, J. H.: The thiosemicarbazones in tuberculosis, Tr. of the Tenth Conference on the Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, Atlanta, Georgia, 1951, p. 120.

(3) BÜNGER, P.: Zur Pathogenese endokriner Syndrome bei der Meningitis tuberculosa, Klin. Wchnschr., 1951, *29*, 338.

# *Notes*

## A STUDY OF CERTAIN AMINO ACIDS AS THEY MAY RELATE TO THE PROBLEM OF HOST RESISTANCE TO TUBERCULOSIS[1]

It is possible that species and organ differences in host resistance and, to a lesser degree, perhaps individual variation in this respect may be attributable to the suitability of a given tissue as a culture medium for the microorganism under study. A knowledge of the chemical constituents of tissue would therefore be valuable from the standpoint of the possible presence of growth stimulators or inhibitors. Of interest also would be the variations induced by fasting, the injection of cortisone, and immunization with BCG, since each of these is known to play a role in resistance to infection. The influence of actual infection on tissue composition would also be important.

The choice, from the infinite number present, of the particular tissue constituents for which analyses should be carried out was difficult. The availability of adequate analytical methods became the primary consideration. In view of the fact that antibodies are nitrogenous substances and that there is evidence that certain amino acids have an inhibitory effect upon the growth of tubercle bacilli *in vitro*[2, 3], total nitrogen and amino nitrogen were included. Since hydroxy amino acids may be of importance in antibody formation[4] and contain the same active chemical groups as the known tuberculostatic compound, para-aminosalicylic acid, the determination of the beta-hydroxy-alpha-amino acid fraction of tissue filtrates was attempted. This was done with the realization that the analytical procedure employed left much to be desired from the standpoint of specificity.

In order to obtain representatives of varying degrees of resistance in both animal species and organs, analyses were carried out on lungs, livers, and spleens from guinea pigs, rabbits, and rats. Organs were removed immediately following the death of the animal, blotted on paper towels, and weighed. Tungstic acid filtrates were prepared by homogenization in Ten Broeck tissue grinders, quantitative transfer to a glass-stoppered graduated cylinder, and dilution to a suitable volume. Duplicate analyses were carried out on aliquots of the protein-free filtrate, for total nitrogen by micro-Kjeldahl, for amino nitrogen by the method of Frame, Russell, and Wilhelmi[5], and for the beta-hydroxy-alpha-amino acid fraction by Boyd's micro modification[6] of the periodate method of Nicolet and Shinn. The removal of sugar preliminary to the latter procedure was accomplished by the use of cupric sulfate and calcium hydroxide.

The values obtained are listed in table 1.

From statistical analyses of these data (comparison between two means) it appears that the most susceptible organ, the lung, had lower normal values for the constituents studied than did either the liver or spleen. Infection with human tubercle bacilli (H37Rv) lowered the values in the organs of all species. Injection of BCG intravenously raised the

[1] Supported by a grant from the Committee on Medical Research of the American Trudeau Society, Medical Section of the National Tuberculosis Association.

[2] Dubos, R. J.: Am. Rev. Tuberc., 1949, *60*, 385.

[3] Marshak, A.: Am. Rev. Tuberc., 1950, *62*, 333.

[4] Staheeva-Kaverzneva, E. D.: J. Gen. Chem. (U.S.S.R.), 1943, *13*, Nos. 9–10.

[5] Frame, E. G., Russell, J. A., and Wilhelmi, A. E.: J. Biol. Chem, 1943, *149*, 255.

[6] Boyd, M. J., and Logan, M. A.: J. Biol. Chem., 1942, *146*, 279. Block, R. J., and Bolling, D.: The Amino Acid Composition of Proteins and Foods, Charles C Thomas, Springfield, Illinois, 1945, p. 396.

values of amino and total nitrogen in organs of the guinea pig and rabbit. The beta-hydroxy amino acid fraction of all three organs of the guinea pig were elevated following BCG injection, as were the amino and total nitrogen values of the liver of the rabbit. Fasting lowered the beta-hydroxy-alpha-amino acid fraction and the amino nitrogen level of all three species.

It is interesting that the most striking changes seen with cortisone treatment were observed in the spleens of rats, where there appeared to be a significant lowering of the beta-hydroxy-alpha-amino acid and amino nitrogen fractions.

TABLE 1

MEAN VALUES OF β-HYDROXY-ALPHA-AMINO ACIDS, AMINO NITROGEN, AND NONPROTEIN NITROGEN OF ORGANS OF DIFFERENT SPECIES

| SPECIES | NUMBER | CONDITION | MG. PER GM. OF FRESH TISSUE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | β-hydroxy-Alpha-Amino-Acid | | | Amino Nitrogen | | | Nonprotein Nitrogen | | |
| | | | Liver | Lung | Spleen | Liver | Lung | Spleen | Liver | Lung | Spleen |
| Guinea Pig | 8 | Normal | 7.14 | .38 | .57 | .70 | .42 | .69 | 1.48 | 1.00 | 1.82 |
| | 17 | Fasting | 4.22* | .29 | .72 | .61* | .31* | .63 | 1.62 | .95 | 1.95 |
| | 13 | Cortisone | 9.04* | .48 | .55 | .66 | .38 | .63 | 1.43 | .95 | 1.71 |
| | 5 | BCG | 13.26* | .54* | .71 | .84* | .41 | .64 | 1.80* | 1.19* | 1.63 |
| | 10 | H37Rv | 5.70* | .35 | .49 | .61 | .29* | .50* | 1.44 | .79* | 1.20* |
| Rabbit | 16 | Normal | 9.07 | .44 | .59 | .55 | .46 | .68 | 1.41 | .91 | 1.49 |
| | 11 | Fasting | 6.67* | .43 | .45* | .53 | .46 | .60* | 1.44 | .95 | 1.52 |
| | 5 | Cortisone | 7.49 | .53 | .42* | .62 | .41 | .62 | 1.87* | .81* | 1.94* |
| | 4 | BCG | 9.66 | .49 | .44* | .69* | .62* | .86* | 1.75* | 1.17* | 1.98* |
| | 5 | H37Rv | 4.97* | .50 | .33* | .47* | .45 | .54* | 1.24* | 1.23 | 1.10* |
| Rat | 19 | Normal | 9.48 | .33 | .53 | .58 | .38 | .70 | 1.57 | .97 | 1.52 |
| | 15 | Fasting | 4.11* | .41 | .25* | .50* | .38 | .31* | 1.33* | .85 | 1.29 |
| | 7 | Cortisone | 9.25 | .33 | .33* | .58 | .37 | .54* | 1.26* | .86 | 1.49 |
| | 4 | BCG | 8.26 | .35 | .34 | .52 | .40 | .66 | 1.53 | .99 | 1.35 |
| | 4 | H37Rv | 9.72 | .25 | .28* | .38* | .31* | .48* | 1.16* | .86 | .99* |

* Statistically significant difference by method of comparison between two means†.

† R. A. Fisher, Statistical Methods for Research Workers, ed. 8., Oliver & Boyd, London, 1941, pp. 116–117.

A direct relationship between host resistance and species differences in the tissue constituents discussed in this report is not necessarily to be anticipated. However, we should like to point out that such species differences do exist and it is quite possible that they may play a role in the ability of the animal to present a more or less favorable environment for the growth of the invading organism.

It should be mentioned that the beta-hydroxy-alpha-amino acid fraction of the liver when calculated as serine accounts for more than 100 per cent of the amino nitrogen found. This indicates the presence of a non-nitrogenous substance not present in the other organs studied, which is being measured by the periodate method employed. We have not as yet identified this substance but we have been able to rule out glucose, glycogen, lactic acid, fructose-6 phosphate, fructose diphosphate, phosphoglyceric acid, and glucose-6 phosphate.

One might explain the finding of lowered values in the infected animals as a result of the poor nutritional state of the animals consequent on the infection. It is more difficult to offer an explanation for the elevation of beta-hydroxy-alpha-amino acid and amino nitrogen values which were observed following immunization of guinea pigs and rabbits with BCG.

These findings cannot at the moment be related to the problem of native resistance of humans to tuberculosis infection. It is clear, however, that some humans react like guinea pigs and rapidly succumb to infection with human tubercle bacilli; others react like rabbits and are able to produce regressive lesions which go on to more or less complete healing; still others react like rats which, although they may harbor the organisms for many months, seldom show clinical evidence of infection and rarely succumb to the disease. One is reminded of individual differences in body chemistry pointed out by Williams[7].

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February 11, 1952

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# *Correspondence*

## PROBLEM OF THE SO-CALLED "GOOD CHRONIC CASE" OF TUBERCULOSIS

*To the Editors of The American Review of Tuberculosis:*

I wish to comment on the report in the May, 1952, issue of the REVIEW by the Committee on Therapy of the American Trudeau Society regarding the problem of the so-called "good chronic" case of tuberculosis. While in general agreement with the statement of the problem and the recommendations for management, it is my opinion and that of a number of my colleagues that considerably more emphasis could have been placed upon the possible suitability of these patients for active therapy. In this connection I refer particularly to prolonged chemotherapy with streptomycin and PAS. In the past one and one-half years we have instituted therapy with prolonged streptomycin and PAS in fifteen "good chronic" cases. Of these, at the present time five have had sputum and gastric cultures negative for tubercle bacilli (averaging ten to twenty cultures) for one year or more, and four have had negative sputum and gastric cultures for six to twelve months. They have, in some instances, been associated with the disappearance of cavities previously of long standing. As yet none of the patients who still yield cultures positive for tubercle bacilli have been shown to have drug-resistant organisms. Despite this fact, two patients with large cavities and sputum persistently positive for tubercle bacilli at the end of one year of combined therapy are now being treated with streptomycin, PAS, and isoniazid in combination.

It is our belief that every patient with active tuberculosis should, unless some contraindication exists, be treated with streptomycin and PAS, with a possible two-year minimum in the most chronic type of case. We no longer believe that we can tell by the appearance upon the roentgenogram of long-standing unchanged caseocavitary and fibrotic disease that a patient is "unsuitable" or will not respond to chemotherapy. It is, of course, true that sputum cultures negative for tubercle bacilli for one or even two years do not constitute a "cure." Nevertheless, it renders these patients noninfectious for prolonged periods of time and, if chemotherapy is continued on an outpatient basis after the first six to twelve months of negativity, may make beds available for more acute cases without at the same time exposing the contacts of these patients to infectious tuberculosis. It is quite possible that at a later date the development of relapses with drug-resistant organisms may lead to a reversal of this policy, but at the present time it would seem to be a hopeful method of attack, with, of course, the possibility of segmental resections in some few suitable cases being held out as a means toward eradication of the most resistant foci.

VETERANS ADMINISTRATION CENTER
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May 14, 1952

Very truly yours,
EMIL ROTHSTEIN

## SURVIVAL OF BACILLI IN TUBERCULOUS LESIONS

*To the Editors of The American Review of Tuberculosis:*

As a pathologist I would like to record some comments relative to a discussion by Dubos which appeared in the REVIEW, issue of May, 1952. The thoughts presented by

Dubos are most provocative. However, they fail to record the relationship between tuberculous lesions and the numbers and distribution of bacilli. It is on this relationship that I wish to comment.

In the experimental animal, bacilli commonly reproduce to considerable numbers up to the appearance of necrosis of the lesion. As the process of necrosis ages, bacilli become fewer in number until it becomes difficult to demonstrate any bacilli. It seems evident that lysis of bacilli in large numbers has taken place. However, it is the fate of bacilli which are not lysed that is most significant. Examination of necrotic lesions which are several months old reveal no bacilli in some and masses of bacilli in colony formation in others—far larger numbers of bacilli than had been observed at any previous date.

Extensive studies of tuberculous lesions in human tissues show that bacilli are extremely difficult to demonstrate in histologic tubercles or in giant cells; that they are seldom found, and then usually singly, in either the peripheral portion of the necrotic zone or in the viable organizing tissue beyond it; that in unsloughed necrotic lesions bacilli may not be found in large portions, whereas in isolated and restricted areas deep within the necrotic tissue, masses of bacilli, sometimes in colony formations, may be found; and that bacilli are most irregularly distributed in "cavity walls", with the largest number present in necrotic tissue that lays in the inner zone of the "wall" and that is totally devoid of viable cellular elements. In recent disseminated foci bacilli may or may not be numerous. This situation probably is related to the initial dosage of bacilli in the new foci. In no instance are they in masses or in colony formations such as one finds in old necrotic lesions. This most irregular distribution of bacilli in lesions is a common phenomenon. It seems evident that under unknown circumstances the bacilli can thrive in wholly necrotic tissue and to a much greater extent than in non-necrotic lesions. Also, it is evident that the necrotic lesion is *the* harborer of bacilli.

In 1950 a study was undertaken at the Veterans Administration Hospital at Sunmount to determine the viability and pathogenicity of bacilli in necrotic lesions that were present in resected lung specimens. In a high percentage of closed necrotic lesions no growth occurred on artificial culture media and guinea pigs failed to show progressive disease, even if bacilli were very numerous in the material used. However, in material taken from open cavities, a high percentage of positive results was obtained. At present it appears that this situation relates primarily to the condition (chemical?) of the tuberculous focus. These results probably will lead to a considerable discussion of whether bacilli in closed necrotic lesions are dead or alive.

I agree with Dubos that it may be most difficult at times to differentiate between "life" and "death" in bacteria. Also, I am in accord with his suggestion that new techniques will be required to solve the problem. I suggest that necrotic tuberculous lesions from persons who have had no chemotherapy should constitute the first material to be studied.

Biggs Memorial Hospital
Ithaca, New York
May 14, 1952

Respectfully yours,
E. M. Medlar

## OXIDATION-REDUCTION DYES FOR THE DETERMINATION OF VIRULENCE OF MYCOBACTERIA IN VITRO

*To the Editors of The American Review of Tuberculosis:*

We have been very much interested by the communication of F. Jean Wilson, Catherine Kalish, and Charles Fish, in The American Review of Tuberculosis, issue of February, 1952, concerning the use of oxidation-reduction dyes for the determination of

virulence of *Mycobacteria in vitro*. We usually use the same test in our laboratory of Hospital Bichat.

We have described in a publication of the *Annales de l'Institut Pasteur* (1950, *79*, 210) the reactions of several strains of *Mycobacteria* to colored indicators of Rh, particularly methylene blue and indophenol blue. In agreement with Bloch, we have shown that the reduction power decreases with virulence. The virulent strains do not possess reducing power, the reduction rate increasing with the lowering of virulence. The BCG strain forms the transition between virulent *Mycobacteria* and the acid-fast saprophytes.

We can admit that these are reactions to the result of powers of adsorption and that the lipids (mycolic acids) play an important part.

It seems, however, hazardous not to take into account the activity of enzyme in the explanation of certain color reactions of *Mycobacteria*. Thus, studying the fixation and decoloration of a methylene blue solution by a *Mycobacterium* well adapted to this kind of reaction, *Mycobacterium phlei*, we could easily dissociate the phase of reduction from the phase of adsorption.

Using the Thunberg tube, we could observe the following facts:

1. During the first minute, a reaction of adsorption takes place, during which the microorganisms appear dark blue in the blue solution. The avirulent *Fleole Bacterium* and the virulent *Mycobacterium tuberculosis* are both colored in the same way.

2. A second period of one and one-half minutes is a period of latency.

3. Finally, the last phase, during one minute, leads to the loss of color of the solution if we deal with the avirulent *Fleole Bacterium*, but no discoloration occurs with the virulent *Mycobacterium tuberculosis*. In thirty minutes the blue of the liquid solution begins to fade and the avirulent germ is less blue. One and one-half minutes later the methylene blue solution is completely colorless. We have already shown (Compt. rend. Soc. de biol., 1952, in press) that the reaction of reduction follows the principal laws of enzymology: effect of pH (optimum, 6.5 to 6.8), of heat (optimum, 37° C.), of inhibitors (alcohol 90°, phenol, ebullition, et cetera).

If the bacteria suspension is first treated at 100° C. during ten minutes or at 70° C. during two hours, the adsorption of color upon the germs may be seen but the second reaction, decoloration, does not happen.

All of these facts show the interest of the work of F. Jean Wilson, Catherine Kalish, and Charles Fish and particularly the great value of their "spot plate virulence test." We are glad to thank these authors for precisions brought about by these tests.

Sincerely,

JEAN DESBORDES
ETIENNE FOURNIER

HOPITAL BICHAT, PARIS
LABORATOIRE DU SERVICE
DU DR. JEAN PARAF

April 23, 1952

# *Books*

DIAGNOSTIC AND EXPERIMENTAL METHODS IN TUBERCULOSIS. Second Edition. By Henry Stuart Willis and Martin Marc Cummings. Pp. 373, Charles C Thomas, Springfield, Illinois, 1952, Price, $10.00.

As suggested by the title and as indicated in the preface, this book was written to fulfill a twofold purpose. First, to give an adequate description and interpretation of the laboratory diagnostic procedures for tuberculosis; and, second, to outline procedures for experimental and investigative work. The authors have succeeded brilliantly in accomplishing their first objective. There long has been a need for a volume which not only gathered together the laboratory diagnostic techniques, but which also adequately explained the principles involved in their use and provided an adequate interpretation of the results of each.

The book begins with a description of the secretions, excretions, and constituents of the body in tuberculosis. These include sputum, gastric content, laryngeal swab, urine, exudates, cerebrospinal fluid, lymph nodes, pus, stool, and blood. The descriptions of these substances are accurate and complete and should be of value to the physician as well as to the laboratory technician. Appended to the first chapter is a section dealing with the two fungus diseases, histoplasmosis and coccidioidomycosis, which frequently closely simulate tuberculosis but which can readily be differentiated from it by laboratory means.

The chapter which follows includes a description of the tubercle bacillus itself, and the differentiation of types and their characteristics. The discussion of the growth of tubercle bacilli on liquid medium is somewhat scant but, in view of the fact that such media have little place in the diagnostic field, this is not a serious limitation. On the whole this chapter, which also includes sections on the pathogenicity, resistance, dissociation, chemistry, and the nature of acid-fastness, provides a solid background for the subsequent detailed consideration of the methods of demonstration of tubercle bacilli.

The demonstration of tubercle bacilli by staining methods, by methods of concentration and cultivation, and by inoculation of animals is covered in detail. These chapters include not only a detailed description of the well-recognized methods and their relative usefulness, but also state the principles clearly. The chapter on culture media is excellent and should serve as a convenient reference source.

Of particular usefulness is a chapter on the nonpathogenic acid-fast bacteria in which the common sources of these troublesome contaminants are detailed and the characteristics of these organisms discussed. This should be very helpful to many laboratory workers who have trouble differentiating these from tubercle bacilli.

In the opinion of the reviewer, the section of the book on diagnostic methods is excellent and accomplishes its purpose admirably.

The second section of the book covers certain related clinical and laboratory methods. Included is a discussion of protective vaccination and the preparation and technique of application of BCG vaccine and the preparation and application of tuberculin. There is also a chapter on antibacterial agents and methods of determining sensitivity, a chapter on serological diagnosis, and one on tuberculin. These are informative and well organized, with the exception of the section on methods for testing antituberculous agents. This is entirely too brief (two and one-half pages) to be of much value, and does not correctly indicate methods which have been found to be most efficient. For example, the impression is gained from reading this section that mice probably are unsuitable for this pur-

pose; whereas, actually, these animals are used more extensively for the experimental chemotherapy of tuberculosis than any other animal species. The lack of adequate references merely emphasizes the deficiencies of this section.

The last section of the book, which is concerned with "some methods of value in the study of tuberculosis experimentally produced," while in the main well done, calls for less enthusiasm. The procedures described unfortunately are limited primarily to the production of tuberculous infection in rabbits and guinea pigs and the nature of the disease in these animals. The coverage of these subjects is excellent, but one is left with the impression that these two species are the only ones that really can be usefully employed. Furthermore, there is insufficient indication of the purposes for which experimental inoculation of animals is used. A more comprehensive discussion of the relative value of rabbits, guinea pigs, mice, and other animals in investigative work in the field of tuberculosis would have been a welcome addition. Although probably beyond the scope of this book, this section would have been far more valuable had it included a discussion and description of the modern techniques employed for the cultivation of tubercle bacilli *in vitro*. There is still too little general appreciation of the fact that tubercle bacilli can now be handled and cultivated almost as easily as the majority of more rapidly growing bacteria and that the results of *in vitro* studies readily can be quantitated.

Although remarkably free of errors, attention should be called to certain misstatements. For example, such statements as "A striking absence of gross lesions follows infection of mice by any method of inoculation" and "Until Dubos added 'Tween 80' to medium, it was an unvarying experience when planting tubercle bacilli onto liquid medium that those bacilli which did not float on the surface did not grow" are inaccurate and, therefore, misleading. Furthermore, to quote Miehe, 1909, to the effect that tubercle bacilli divide every six hours ignores more recent work which shows conclusively that the generation time of virulent tubercle bacilli in the better liquid media is between fourteen to twenty hours. The quoted generation time of six hours actually corresponds to that of many acid-fast saprophytes.

The book on the whole is an excellent one and should prove a valuable addition to the literature on tuberculosis; however, it should be emphasized that it is primarily a practical laboratory guide to be used as an aid in the laboratory diagnosis of tuberculosis. It should not be considered a highly scientific treatise on the bacteriology of tuberculosis.

GUY P. YOUMANS
Chicago, Illinois

ANTIBIOTIC THERAPY. By Henry Welch and Charles N. Lewis, foreword by Chester S. Keefer. Pp. 562, The Arundel Press, Inc., Washington, D. C., 1951, Price, $10.00.

This is a well-written and remarkably up-to-date book about antibiotic therapy. The first half summarizes bacteriologic and pharmacologic data, and contains much valuable information concerning absorption and excretion and dosage forms. Brief biographical sketches of the discoverers of the various antibiotics add to the interest of this section. The second half deals with the therapy of specific infectious diseases. The current literature is cited, and there is a bibliography at the end of each chapter.

One can find little to criticize in this excellent book. In writing it the authors were obviously aware of much unpublished work, and were therefore able to include more current data than is usually possible in such a fast-moving field. The section on tuberculosis is particularly good, and presents a sound evaluation of methods and results of therapy of all forms of the disease.

The first half of the book contains many facts about the discovery, development, and

nature of antibiotics that are not available elsewhere. Because of their association with the Food and Drug Administration, the authors are uniquely qualified to present this information. Although less outstanding, the clinical sections are also very good. Particularly striking are the frequent summaries of the information which is presented. This book will be useful for the specialist in the field of antibiotics, as well as for the busy practitioner seeking aid in therapy. To remain up to date, frequent revisions will of course be necessary.

WILLIAM M. M. KIRBY
Seattle, Washington

COMMUNITY HEALTH EDUCATION IN ACTION. By Raymond S. Patterson and Beryl J. Roberts. Pp. 346, The C. V. Mosby Company, St. Louis, Missouri, 1951, Price, $4.50.

This book is likely to be useful to the health officer, the health nurse, the sanitarian, and the many workers whose responsibilities are within a community health department. Attention is devoted to means by which known material can be made available and memorable to those in need. Details of a health educational program are discussed at moderate length. For those requiring more information, there are suggestions for reading concluding each chapter.

The book is a practical manual affording the reader an integration of experience, techniques, and communication devices valuable in group education. The material is presented simply, is well organized, and is in an easy, pleasant style.

CARL BERNTSEN
New York, N. Y.

## BOOKS RECEIVED

INTERNAL MEDICINE. Fifth edition. Edited originally by John H. Musser; this edition edited by Michael G. Wohl. Pp. 1,563, with illustrations, Lea & Febiger, Philadelphia, 1951, Price, $15.00

THE THORACIC SURGICAL PATIENT. By Lew A. Hochberg, Pp. 365, Grune & Stratton, Inc., New York, 1952, Price, $8.75

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THE AURICULAR ARRHYTHMIAS. By Myron Prinzmetal, Eliot Corday, Isidor C. Brill, Robert W. Oblath, and H. E. Kruger, and associate authors. Pp. 398, with illustrations, Charles C Thomas, Springfield, Illinois, 1952, Price, $16.50

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MASS BCG VACCINATION IN ISRAEL, 1949–1950, with Special Reference to Statistics on Tuberculin Testing and BCG Vaccination. Pp. 34, The International Tuberculosis Campaign, UNICEF EMRO, 24 Rue Borghèse, Neuilly-sur-Seine, France, 1951.

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MERCK INDEX. Sixth edition. Pp. 1,165, Merck & Co.. Inc., Rahway, New Jersey, 1952, Price, $7.50 regular edition; $8.00 thumb-index edition.

CAUSES AND PREVENTION OF TUBERCULOSIS. By Brice R. Clarke. Pp. 288, The Williams & Wilkins Company, Baltimore, Maryland, 1952, Price, $6.50

THE FIGHT AGAINST TUBERCULOSIS. By Francis Marion Pottenger. Pp. 276, Henry Schuman, New York, N. Y., 1952, Price, $4.00

*American Trudeau Society*

**Medical Section of the National Tuberculosis Association**

## NOTES

### REQUEST FOR DATA ON TOXICITY OF ISONIAZID AND IPRONIAZID

Isoniazid (isonicotinic acid hydrazide) and iproniazid (1-isonicotinyl-2-isopropyl hydrazine) are being given to many patients with tuberculosis throughout the United States and in many other countries. Toxic reactions to both drugs have been reported and, undoubtedly, more will occur. However, it is unlikely that any single investigator will observe enough of these toxic reactions in the next few months to warrant publication. The Committee on Therapy of the American Trudeau Society is anxious to collect reports of these toxic reactions, so that a consolidated report can be made available to the medical profession as soon as possible.

Any physician who is willing to submit such reports to the Committee is asked to send a brief case report to the Committee on Therapy, American Trudeau Society, % Dr. D. T. Carr, 102–110 Second Avenue Southwest, Rochester, Minnesota. The report should include the patient's name or initials, his age, sex, race, diagnosis, weight, the drug administered and its dosage, the duration of treatment, and a list of other drugs being given at the same time. The toxic reaction should be described fully, together with information as to its recurrence if the same drug was given later.

Copies of the consolidated report will be sent to all contributors, but will not be published in such a way as to preclude individual publication.

### ANNUAL MEETING OF THE AMERICAN TRUDEAU SOCIETY—1953

The American Trudeau Society, medical section of the National Tuberculosis Association, invites the submission of abstracts of scientific papers for presentation at its next annual meeting in Los Angeles, California, May 18 to 22, 1953, at the Hotel Statler.

Full information can be obtained by writing to the Chairman of the Medical Sessions Committee, American Trudeau Society, 1790 Broadway, New York 19, N. Y.

### ANNUAL MEETING OF THE EASTERN SECTION OF THE AMERICAN TRUDEAU SOCIETY

The Eastern Section of the American Trudeau Society will hold its annual meeting on Friday and Saturday, October 31 and November 1, 1952, in Philadelphia, Pennsylvania. Headquarters for the meeting will be at the Bellevue-Stratford Hotel in that city. Members of the Society who wish to present papers are urged to communicate promptly with the Chairman of the Program Committee, Dr. Katharine R. Boucot, 311 Juniper Street, Philadelphia, Pennsylvania.

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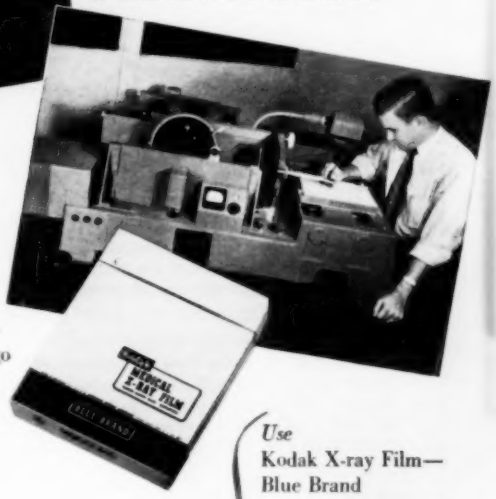


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by

G. S. Wilson, MD, FRCP, MRCS, DPH, *Director*, Public Health Laboratory Service, Medical Research Council

J. W. S. Blacklock, MD, FRFPS, *Professor* in Pathology, St. Bartholomews Hospital Medical College, London

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1. Disler, M.: Dosage and Blood Levels of Para-Aminosalicylic Acid, Schweiz. Ztschr. Tuberk. 7:6 (1950).

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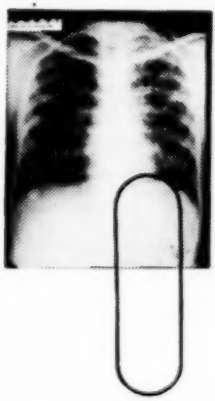

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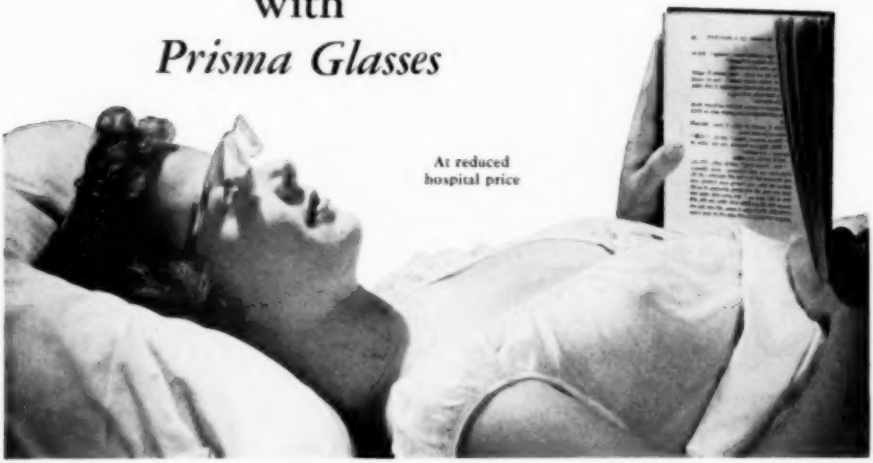


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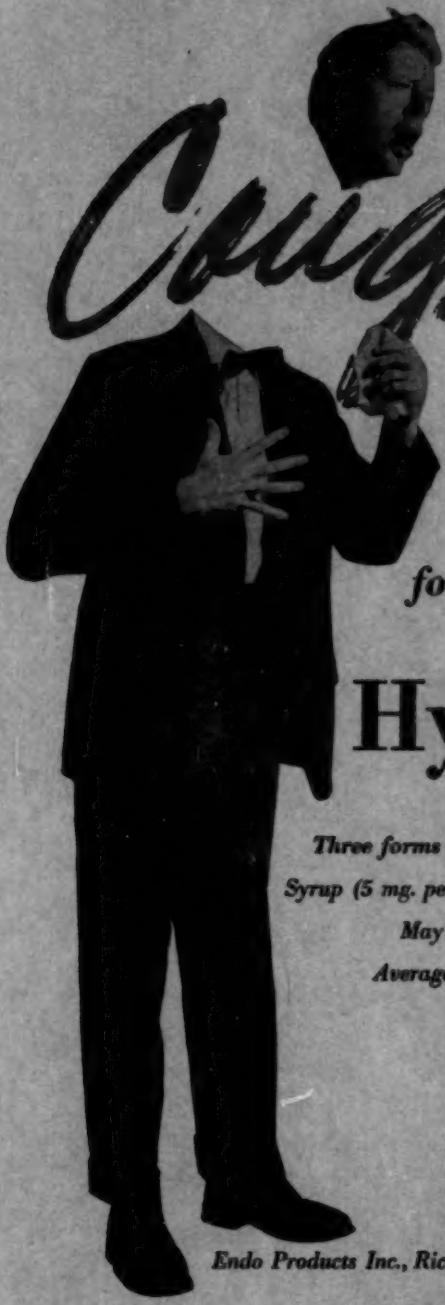

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